The Role of Serine Protease HTRA1 in Bone Formation and Regeneration

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Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

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Zürich 2017

Aloha.

Summary

The role of mammalian high-temperature requirement protease A1 (HTRA1) in MSC osteogenic differentiation remains highly controversial. Numerous studies using different cell lines have characterized HTRA1 as either a positive or negative regulator of osteogenesis and matrix mineralization. In order to investigate this further, we initially assessed the role of HTRA1 in MSC osteogenesis *in vitro* using the C3H10T1/2 mouse mesenchymal stem cell line. We asked the question whether the ablation of *Htra1* using a short hairpin RNA could affect the BMP2-induced differentiation of C3H10T1/2 cells into mineralizing osteoblasts. We demonstrated that loss-of-function of HTRA1 enhanced the expression of several osteogenic gene markers and significantly increased matrix mineralization of BMP-2-treated C3H10T1/2 cells as determined using Alizarin Red S staining. Additionally, these effects were accompanied by an increase in the expression of adipogenic gene markers and a decrease in chondrogenic gene markers. Increases in Oil Red O staining further confirmed adipogenesis to be enhanced in HTRA1-deficient C3H10T1/2 cells. We also investigated the role of HTRA1 in bone formation *in vivo* using mice deficient in the *Htra1* gene. However, in contrast to our *in vitro* findings, HTRA1 loss in 16-week-old mice failed to induce any significant alterations in bone formation as determined by micro-CT analysis. Furthermore, cartilage callus formation and bone repair were observed to proceed normally following femur osteotomy. By comparison, micro-CT analysis of intact femurs from 52-week-old mice revealed that bone structure was better preserved in *Htra1*-knockout mice than age-matched wild-type controls. Immunohistochemical analysis of paraffin embedded tissue sections from osteotomized femurs identified HTRA1 in wild-type mice only, and HTRA3 in both wild-type and *Htra1*-knockout mice. Taken together, our findings further identify HTRA1 as a potent regulator of the multilineage differentiation potential of MSCs, and provide evidence to suggest that although HTRA1 does not appear to influence bone development and regeneration beyond the *in vitro* system, it may contribute to the aging bone phenotype in mice. Additionally, possible functional redundancy might exist between the HtrA paralogs, whereby loss of HTRA1 may be compensated for by HTRA3.

Zusammenfassung

Die Rolle der hoch Temperatur abhängigen Protease A1 (HTRA1, high-temperature requirement protease A1) in der osteogenen Differenzierung von Mesenchymalen Stammzellen (MSC`s) wird kontrovers diskutiert. Es liegen zahlreiche Studien vor, welche HTRA1 in unterschiedlichen Zelllinien als positiven oder negativen Regulator der Osteogenese und Matrixmineralisierung beschreiben. Um die Rolle von HTRA1 auf diesem Gebiet weiter zu analysieren, untersuchten wir zunächst seine Effekte auf die Osteogenese *in vitro* in einer mesenchymalen Stammzelllinie der Maus (C3H10T1/2). Wir stellten die Frage, ob die Hemmung des *Htra1*-Gens mittels “short hairpin RNA“ die durch BMP-2 induzierte Differenzierung von C3H10T1/2-Zellen in mineralisierende Osteoblasten beeinflusst. Wir konnten zeigen, dass C3H10T1/2-Zellen ohne funktionales HTRA1 eine gesteigerte Expression knochenbildender Genmarker und eine erhöhte Matrixmineralisierung aufweisen. Begleitet wurden diese Effekte durch eine höhere Expression bestimmter Gene des Fettstoffwechsels und verminderter Expression von Genen des Knorpelstoffwechsels. Weiterführende Versuche mit Oil Red O zur Färbung von Triglyceriden zeigten ebenfalls eine erhöhte Adipogenese in C3H10T1/2 Zellen ohne HTRA1. Wir untersuchten ausserdem die Rolle von HTRA1 während der Osteogenese *in vivo* in Mäusen, welche das Gen für *Htra1* nicht in sich tragen. Im Gegensatz zu unseren *in vitro* Untersuchungen zeigte der Mangel an HTRA1 in Mikro-CT-Analysen 16-Wochen alter Mäuse keine wesentlichen Änderungen in der Knochenbildung. Zudem folgten nach Osteotomie des Oberschenkelknochens sowohl die Kallusbildung als auch die Knochenregeneration der normalen Entwicklung. Die Femora von 52-Wochen alten Knock-out Mäusen zeigten eine besser erhaltene Knochenstruktur als die Oberschenkelknochen von Kontrolltieren vergleichbaren Alters. Die immunhistochemische Analyse von Gewebeschnitten der Osteotomiedefekte zeigte HTRA1 ausschliesslich in der Kontrollgruppe, HTRA3 sowohl in Wild-Typ als auch *HtrA1*-Knock-out Mäusen.
Zusammengefasst bestätigen unsere Ergebnisse, dass HTRA1 ein einflussreicher Regulator der multipotenten Differenzierung von MSC`s ist. Des Weiteren erbringen sie den Nachweis, dass HTRA1 zur Knochenalterung in Mäusen beiträgt, auch wenn es *in vitro* keinen Einfluss auf die Knochenentwicklung und Regeneration zu haben scheint. Zusätzlich scheinen

die HtrA Gene paralog zueinander zu sein, so dass der Verlust an HTRA1 durch funktionale Redundanz von HTRA3 kompensiert werden kann.

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Abbreviations

Akt/PKB – protein kinase B

ATP – adenosine triphosphate

BLC – bone lining cells

BM – bone marrow

BMD – bone mineral density

BMP – bone morphogenetic proteins

BMT – bone marrow transplantation

Col1α1 – type 1 collagen subunit alpha 1

DegP – degradation of extracellular proteins

Dlx5 – distal-less homeobox 5

DMP1 – dentine matrix protein 1

ECM – extracellular matrix

EGFR – epidermal growth factor receptor

ESC – embryonic stem cell

ESR – oestrogen receptor 1

EVs – extracellular vesicles

FGF – fibroblast growth factor

FOP – fibrodysplasia ossificans progressive

GC – glucocorticoid

HA – hydroxyapatite

HPP – Hypophosphatasia

HSC – hematopoietic stem cells

HTRA – high temperature requirement A

IAP – inhibitor of apotosis

IGFBD – insulin-like growth factor binding domain

IVD – intervertebral disk

KM – Kasal-type inhibitor module

M-CSF – macrophage colony stimulating factor

MMP – matrix metalloproteinase

MSC – mesenchymal stem cell

NFATc1 – nuclear factor of activated T-cells cytoplasmic 1

OA – osteoarthritis

OP – osteoporosis

OPG – osteoprotegrin

Osx – osterix

PC1 – polycystin 1

PC2 – polycystin 2

PD – protease domain

PTH – parathyroid hormone

RA – rheumatoid arthritis

RANKL – receptor activator of nuclear factor κ-B ligand

RT-qPCR – reverse-transcription quantitative polymerase chain reaction

RUNX2 – Runt-related transcription factor

SOST – sclerostin

SOX9 – sex determining region Y box 9

SP – signal peptide

SVF – stromal vascular fraction

TGFβ – transforming growth factor β

TRAP – tartrate resistant acid phosphatase

VDR – vitamin D receptor

Wnt – Wingless-type MMTV integration site

1. Introduction

1.1. Stem Cells

Stem cells were discovered in the 1950s during studies undertaken into testicular teratoma formation in mice [1]. Tumours originating from spontaneous teratomas in mice testes grew into a variety of embryonic and adult tissues not usually specific to mice testes. Stevens *et al.* (1967) demonstrated that these tumours were the result of heterogeneously differentiated primordial germ cells in foetal testis [1,2] and characterised them as 'pluripotent embryonic cells'. These cells exhibited the ability to generate differentiated cells while conserving a group of undifferentiated cells [3]. A number of teratomas were dissociated, and a single cell was isolated and transplanted into a mouse embryo [4]. This transplanted single cell was multipotent and formed tumours composed of both differentiated (by heterogenic degrees) and embryonic tissues. While transplantation of this single cell into adult mice was lethal, other studies investigated whether a single-cell transplantation of teratocarcinoma into mice blastocysts was viable [5,6]. The transplanted cell did not react as a malignant cell; instead, it contributed to the normal development of the blastocyst and remained under controlled proliferation. The result of this blastocyst manipulation was a chimeric mouse with tissues mostly derived from the injected teratoma cell. Subsequently, teratoma cells were considered to be embryonic stem cells (ESCs) rather than abnormal tumorigenic cells. More than twenty years of intensive research was required for the concept of embryonic stem cells to become established [7].

1.1.1. The Stem-cell Niche

The idea of such a niche was introduced for the first time in 1978 [8]. At the time, it was considered to be a cell-cell dependent interaction, with each cell controlling the other's future development [9]. The discovery of germ-line stem cells in *Drosophila melanogaster* initiated the understanding of the niche's molecular mechanisms responsible for cell differentiation, tissue homeostasis and regeneration.

Body tissue formation is the result of extensive remodelling processes; it is dependent on stem cells' ability to migrate, proliferate and differentiate on cue.

Depending on the stimuli and tissue type, stem cells' requirements and turnover frequency differ. As a result, stem cells may remain dormant for varying periods of time. They reside in specific body locations, creating a favourable microenvironment for cell quiescence called 'niches' [10]. Within a niche, stem cells remain undifferentiated due to cell-to-cell signalling. However, the differentiated progeny can leave the niche to colonise and establish new tissues. Communication between the differentiating progeny and the stem-cell pool is then established in order to control cell determination. The manner in which stem-cell properties are maintained is specific to the species and niche type [11].

1.1.2. Hierarchy

The hallmark of stem cells is their potential to differentiate into distinct cell types in a multicellular organism. The **totipotent** fertilized egg (zygote) exhibits the highest potential of differentiation due to its ability to generate all cell types that constitute the three germ layers (endoderm, mesoderm, ectoderm) and the placenta, and eventually create the entire organism. Another feature of totipotent stem cells is the ability to self-renew without losing total potency. Thus, the zygote has the capacity to divide and generate totipotent replicas during the early stages of foetal development [12].

At the blastocyst stage, the cells give rise to **pluripotent** ESCs, which preserve the characteristics of a totipotent stem cell to develop into all cell types but not a complete organism [13].

In addition to the terminally differentiated and specialised cell types in a given tissue, a pool of germ layer-specific undifferentiated quiescent cells often exists; these cells preserve a **multipotent** capacity to differentiate into various cell types of their resident germ layer. For example, the mesoderm is the result of the differentiation of hematopoietic and multipotent stromal cells/mesenchymal stem cells (MSCs) [14].

Stem cells can also be predetermined to differentiate into a specific tissue, meaning that they exhibit **oligopotency** capacities. For example, osteoblasts and preadipocytes have restricted capacity to become another cell type [15].

Once a cell is fully differentiated and cannot differentiate further, it is considered to be **nulipotent** [16].

1.1.3. Mechanisms of Differentiation

The emerging awareness of stem cell potency, together with the growing need for enhanced therapeutic technologies to regenerate diseased or degenerated tissues, has led to increased interest in the use of stem cells for applied human tissue and organ regeneration. Subsequently, research on the processes that govern stem cell differentiation has increased in the last decade and provided fundamental insights into the regulatory processes controlling cell fate.

Initially, it was thought that the mechanisms governing stem-cell homeostasis were mediated through a unidirectional process in which stem cells differentiated into desired tissues following a specific activation stimulus. However, stem cells exhibit an expanded repertoire of differentiation mechanisms that confer them considerable plasticity; this repertoire is categorized in four primary mechanisms (Figure 1):

- "**Dedifferentiation**" is a cell's ability to lose its specialization and revert to a more simplified form. Within the same lineage, differentiated cells can return to a less mature differentiation stage (Figure 1, dashed green arrows).
- **Trans-determination** is a stem cell's ability to switch from one lineage to another and then differentiate into a cell type different from the original (Figure 1, light brown arrows).
- **Trans-differentiation** is the conversion of a differentiated cell into completely unrelated tissues [11] (Figure 1, blue arrow).
- **Reprogramming** is when a cell is returned to its most pluripotent stage, enabling its differentiation into any type of tissue (Figure 1, pink arrow).

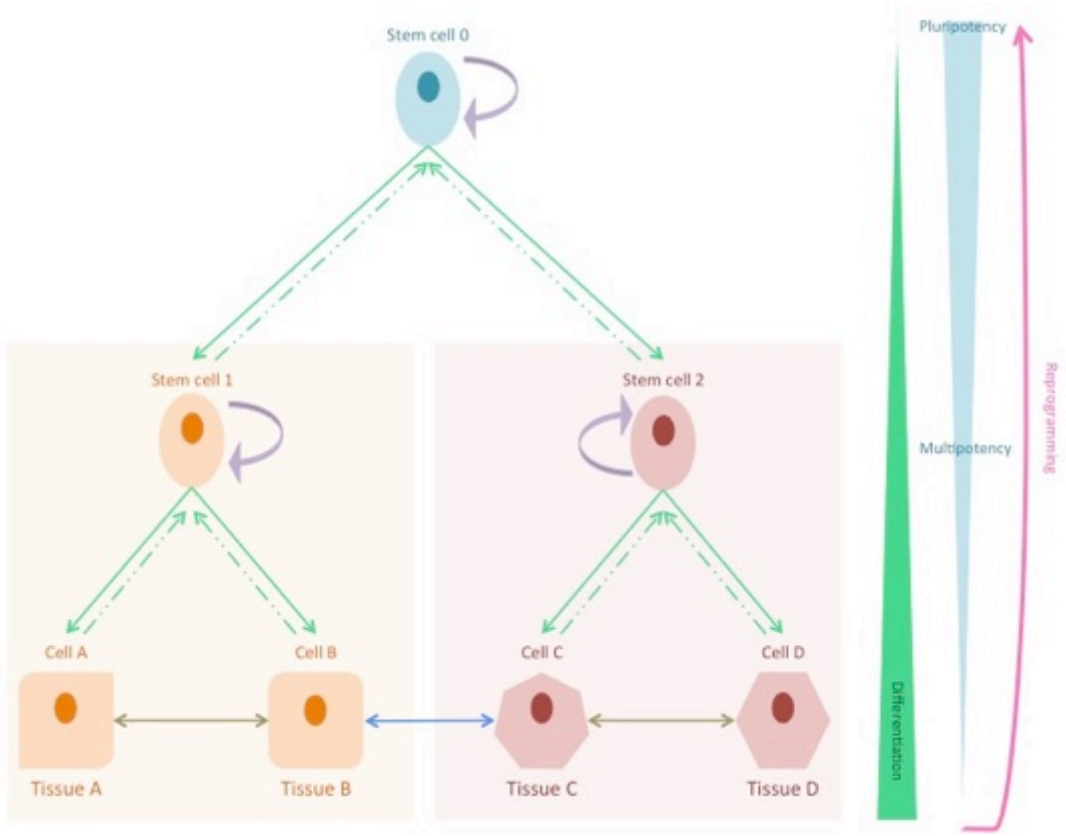

Figure 1: Stem Cell Plasticity
Stem cells have the capacity to self-renew (purple arrows). Pluripotent stem cells can differentiate into any cell type, while multipotent stem cells are limited to certain lineage commitments (solid green arrows). Stem cells can also dedifferentiate to a less mature stage (dashed green arrows), trans-determine (light brown arrows), trans-differentiate (blue arrow) or reprogram (pink arrow).

1.1.4. Adult Versus Embryonic Stem Cells

Embryonic stem cells are a useful tool to study development and tissue homeostasis disorders. However, the use of ESCs in human-based biomedical research has raised ethical issues due to the cells' origin [17]. Consequently, their application as therapeutic tools has remained extremely limited. The growing need to find an alternative to this working model for cell-based regenerative medicine has resulted in a shift towards multipotent adult stem cells such as MSCs [18,19]. The advantage of these adult stem cells is that they are present in the body throughout an organism's lifetime. In addition to MSCs, the body provides other adult stem cells including the following:

- Hematopoietic stem cells (HSCs) that are found in the red bone marrow. Their differentiation promotes the establishment of myeloid (erythrocytes, thrombocytes, monocytes and macrophages) and lymphoid (lymphocytes and natural killer cells) lineages [20,21].

- Mammary stem cells originate from the mammary gland and have exhibited multipotent and totipotent features [22].
- Intestinal stem cells are located in the gut's epithelium. They regenerate the epithelium throughout the organism's life [23,24].

1.2. Mesenchymal Stem Cells (MSCs)

MSCs constitute a population of multipotent adult progenitors with a fibroblast-like morphology. They can differentiate into several mesenchymal lineages and, therefore, maintain organ homeostasis [25]. They were discovered in 1966 through bone marrow (BM) transplant experiments in mice [26]. It was discovered that after transplantation, BM cells could differentiate into reticular and bone tissues. This finding suggested that BM cells might have a common progenitor. Further studies demonstrated that MSCs exhibit an ability to self-renew and differentiate into specific cell types, with the potential to replace, regenerate or rejuvenate injured tissues [25,27,28]. As multipotent progenitors, their potential for differentiation remains limited to cartilage, bone, fat and muscle. Nevertheless, they quickly began to attract interest in the scientific community as a promising alternative to ESCs.

1.2.1. Overview

Numerous sources of MSCs have been identified, including BM, placenta, trabecular bone [29], adipose tissue [30], skeletal muscle [31], blood, lungs, umbilical cord [32,33], and teeth [34].

MSCs exhibit the stem cell characteristic of self-renewal; however, their mechanism of differentiation is not yet fully understood, and their stability *in vitro* remains highly variable [29]. Additionally, MSCs can be induced to differentiate into different cell types derived from the embryonic mesoderm, such as osteoblasts, chondrocytes, and adipocytes (Figure 2). MSC lineage commitment is dependent on the expression of specific genes. However, certain sets of genes are commonly expressed among all mesenchymal lineages. Certain factors have been identified as essential to MSC differentiation, directing the cells along a specific cell lineage and maintaining their terminal phenotype. Most notable in this respect are transforming growth factor β (TGF-β), collagens, proteoglycans and members of the Wingless-type MMTV integration site (Wnt) [35].

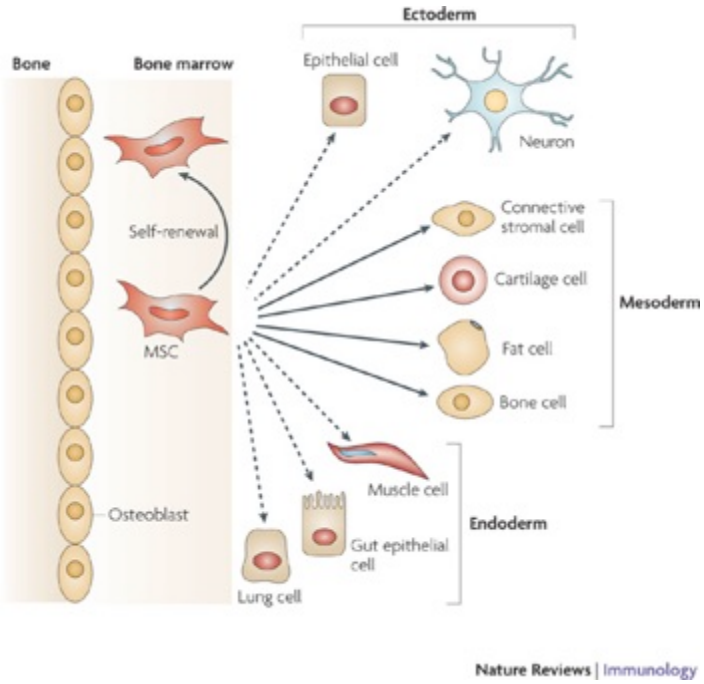

Figure 2: MSC Differentiation
Located in the bone marrow, MSCs can self-renew and differentiate into several cell types of the mesodermal lineage (black arrows). *In vitro*, MSCs have also been shown to trans-differentiate and give rise to ectoderm or endoderm lineages [36].

1.2.2. Adipogenesis

Adipose tissue can be classified into two main types that have antagonist functions, white adipose tissue (WAT) and brown adipose tissue (BAT) [37,38]. White adipose tissue represents the compartment storing excess of energy under triacylglycerol (TAG). Adipocytes are the major components of WAT, and they can store considerable amounts of TAG. Triacylglycerol can be hydrolysed into fatty acids to sustain organs if the body faces a prolonged period of energy

deprivation [39]. BAT is composed of brown adipocytes containing many more lipid droplets than adipocytes from WAT. The brown colour of these tissues is conferred by the presence of numerous mitochondria containing iron. Brown adipose tissue's main function is thermoregulation of the body.

Adipogenesis is a two-step process through which MSCs differentiate first into preadipocytes, and then into adipocytes filled with lipids. This process occurs during excessive energy intake and glucose uptake [40]. The efficiency of adipogenesis is dependent on cell density and distribution, and is principally governed by the activities of the transcription factor CAAT/enhancer-binding protein (C/EBP) and peroxisome proliferator-activated receptor γ (PPARγ) ligand (Figure 3) [41]. Additionally, several other pathways also play a role in adipocyte formation.

Firstly, bone morphogenetic protein (BMP) signalling was identified as a promoter of adipogenesis through *BMP2* and *BMP4* [42]. Additionally, Wnt signalling was found to initiate adipogenic commitment through its canonical pathway where β-catenin degradation is inhibited, thereby allowing for the transcription of adipogenic genes and the initiation of MSC adipogenic commitment [43]. At later stages, when preadipocytes are committed to becoming adipocytes, the Wnt canonical pathway actually acts to inhibit adipogenesis, and instead promotes other mesodermal fates. Hedgehog (Hh) signalling has also been found to have an inhibitory effect on adipogenesis [44], although the underlying mechanisms through which Hh regulates adipogenesis are not well understood.

Due to its endocrine and paracrine capacities, adipose tissue is implicated in several processes, such as glucose metabolism, appetite, immunological response and inflammatory response [45].

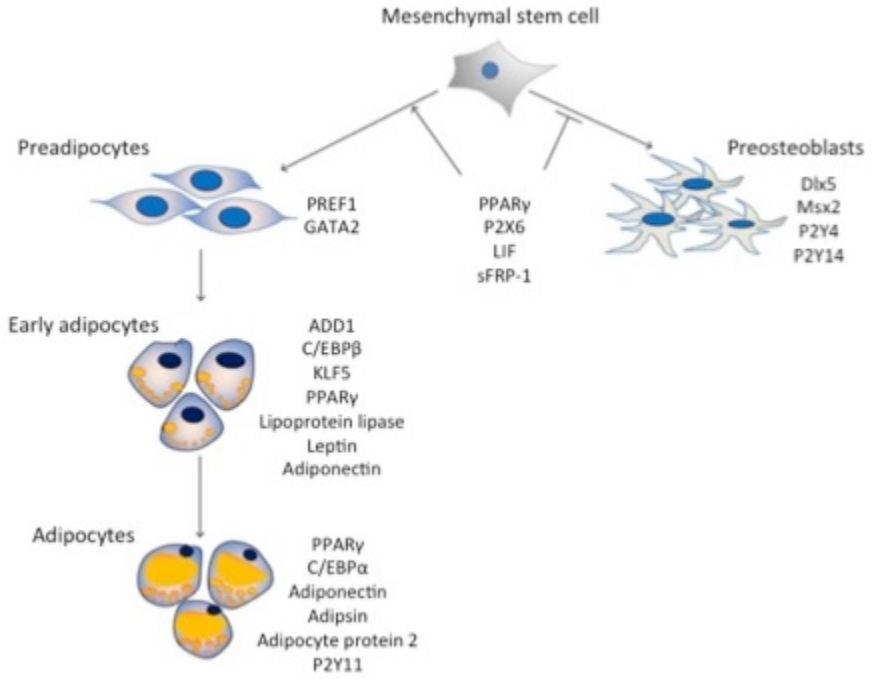

Figure 3: Commitment of MSCs Toward an Adipogenic Fate
PPARγ drives MSCs to differentiate into adipocytes. Preadipocytes are characterised by the expression of preadipocytes factor 1 (Pref-1) and GATA binding protein 2 (Gata2). Preadipocytes become early adipocytes and a switch in the expression of adipogenic markers is marked: adipocyte determination and differentiation (ADD1), CAAT/enhancer binding protein β (C/EBPβ), Kruppel-like factor (KLF5), PPARγ, lipoprotein lipase (LPL), leptin and adiponectin. Mature adipocytes are the terminal stage, expressing PPARγ, C/EBPα, adiponectin, adipocyte protein 2, adipsin and purinergic receptor (P2Y11) [46].

1.2.3. Chondrogenesis

Chondrocytes produce articular cartilage, a layer of specialized connective tissue found at the ends of bones in joints. Articular cartilage not only diminishes friction during articulation by providing a smooth and lubricated surface, but also acts as a buffer zone, transmitting loads to the subchondral bone [47]. Articular cartilage can be subdivided into 2 phases:

- The **fluid phase** is composed of 80% of water and inorganic ions such as calcium, sodium, chloride and potassium.
- The **solid phase** consists of a porous and permeable extracellular matrix (ECM) composed of collagen and elastin fibres, proteoglycans and glycoproteins [48].

The major fibrillar components of the ECM are collagens; collagens II, III, IX, VI and XI are found in the hyaline cartilage. In the calcified layers, the hypertrophic cartilage abundantly expresses collagen X. The non-collagenous components,

including glycosaminoglycans (GAGs), proteoglycans and glycoproteins, are bound to the fibrillar elements in the ECM.

Chondrogenesis results from the condensation of MSCs; although detailed mechanisms driving chondrogenesis are still largely unknown, some key players have been identified.

Sonic hedgehog (Shh) signalling is expressed by the sclerotome which is the mesenchymal region originating from the ventral region during development [49]. Shh induces Snail, which promotes the epithelial-mesenchymal transition of the sclerotome [50]. Moreover, Shh is responsible for induction of the sclerotome, and this process requires the repression of BMP signalling. Shh initiates the expression of the transcription factor sex-determining region Y box 9 (*SOX9*), which is crucial for MSC differentiation toward chondrocytes. When the sclerotome differentiates into cartilage at later stages, Shh signalling is no longer required, and its expression pattern is decreased [51]. As a result, BMP signalling takes over and in turn contributes to maintaining *SOX9* expression [52,53].

The canonical pathway of Wnt signalling prevents cartilage formation in the limb bud. β-catenin represses the expression of *SOX9* since the conditional loss of β-catenin correlates with an increase in *SOX9* expression, preventing progenitors from differentiating into osteoblasts [54,55]. Additionally, it has been demonstrated that β-catenin-mediated *SOX9* repression occurs through the DNA methylation of the *SOX9* promoter. This repression can however be counteracted by fibroblast growth factor (FGF) signals [56].

Differentiated chondrocytes can be subdivided into two populations. The first population includes round and low proliferating chondrocytes expressing SOX9 at the distal end of the proliferation centre. Well-aligned chondrocytes expressing SOX9 and scleraxis (SCX) make up the second population. These progenitors mature, undergo hypertrophy, and ultimately partake in the formation of new bone [57]. Long considered as controversial, TGF-β signalling plays a critical role in chondrogenesis. Indeed, while TGF-β is not needed for the development of the first population of chondrocytes, its intervention is central to the commitment of the second group of chondrocytes to bone formation [58]. In these chondrocytes, TGF-β signals through SMAD2 and SMAD3, which interact with SOX9 to ultimately increase SOX9 expression (Figure 4) [59].

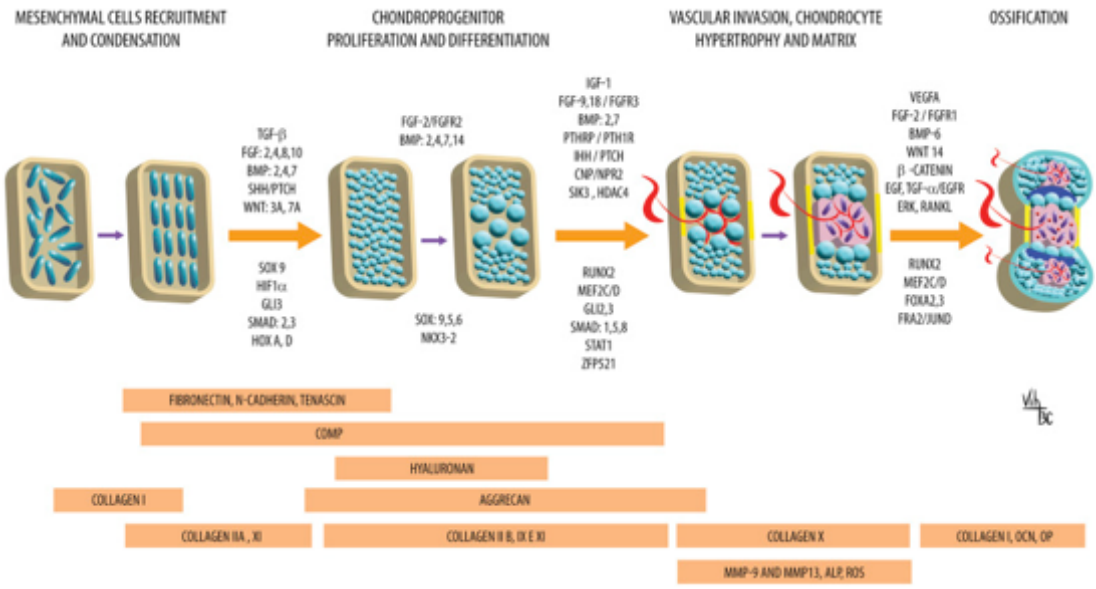

Figure 4: MSC Commitment Toward a Chondrogenic Fate
A schematic overview of the different pathways involved in the regulation of chondrogenesis mediating cartilage and bone formation [47].

1.2.4. Osteogenesis

Osteogenesis refers to differentiation of MSCs towards bone-producing osteoblasts (Figure 5). Two major mechanisms are involved in bone formation, namely intramembranous and endochondral ossification. Intramembranous ossification involves the direct formation of bone by MSC-derived osteoblasts, whereas in endochondral ossification, cartilage is first formed, which later becomes calcified by invading osteoblasts. These two mechanisms occur at various stages of human life and give rise to different types of bones. The next paragraph provides a detailed description of osteogenesis.

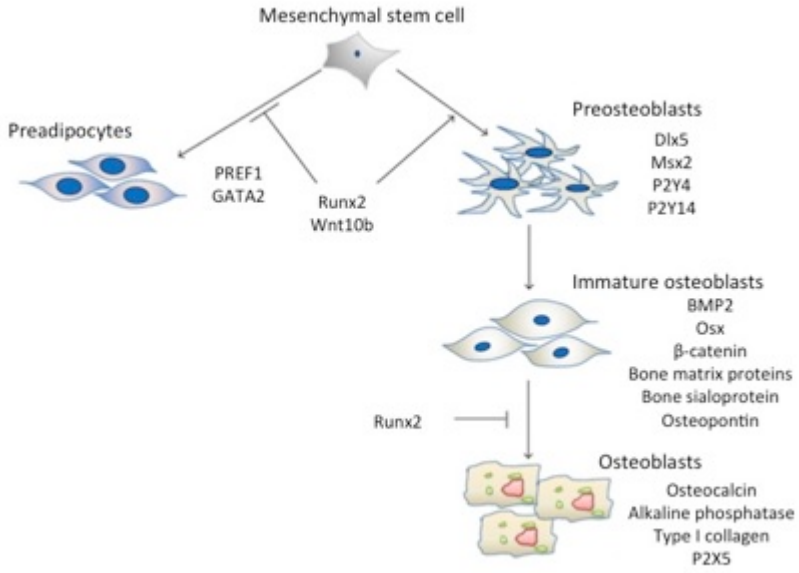

Figure 5: MSC Commitment Toward an Osteogenic Fate
Runt-related transcription factor 2 (*RUNX2*) promotes MSC differentiation into osteoblasts and inhibits adipogenesis. Preosteoblasts express specific osteogenic markers such as distal-less homeobox (*DLX5*) and msh homeobox homologue 2 (*MSX2*). The next step is characterized by the expression of *BMP-2*, osterix (*OSX*), bone sialoprotein, osteopontin and β-catenin. The terminally differentiated osteoblasts express alkaline phosphatase, osteocalcin and type I collagen [46].

1.3. Osteogenesis and Bone

The mammalian skeleton is a combination of a variety of cell lineages that reflect evolution over millions of years. During embryogenesis, MSCs derived from prechordal, paraxial or lateral plate mesoderms migrate to sites where bone is produced. Once at these sites, cells start to condense. The resulting high cellular density constitutes the appropriate environment to initiate cell differentiation. At this stage, two different processes of osteogenesis are conceivable for the MSCs. They either differentiate directly into osteoblasts, or first become chondrocytes and then are replaced by invading osteoprogenitors, or undergo osteogenesis to become osteoblasts themselves [60]. The first mechanism is called intramembranous ossification, and it occurs in the membranous cranium, clavicle and flat bones. The second mechanism, endochondral ossification, is responsible for the development of the basal and posterior parts of the skull, the axial and appendicular skeleton, and long-bone formation.

1.3.1. Bone Development

In certain body areas such as flat bones of the cranium, MSCs are organised in clusters and following their differentiation into osteoblasts, can generate a matrix rich in type I collagen [61]. This intramembranous ossification is limited to embryonic development and superseded by endochondral ossification during late stages of pregnancy and after birth (Figure 6A).

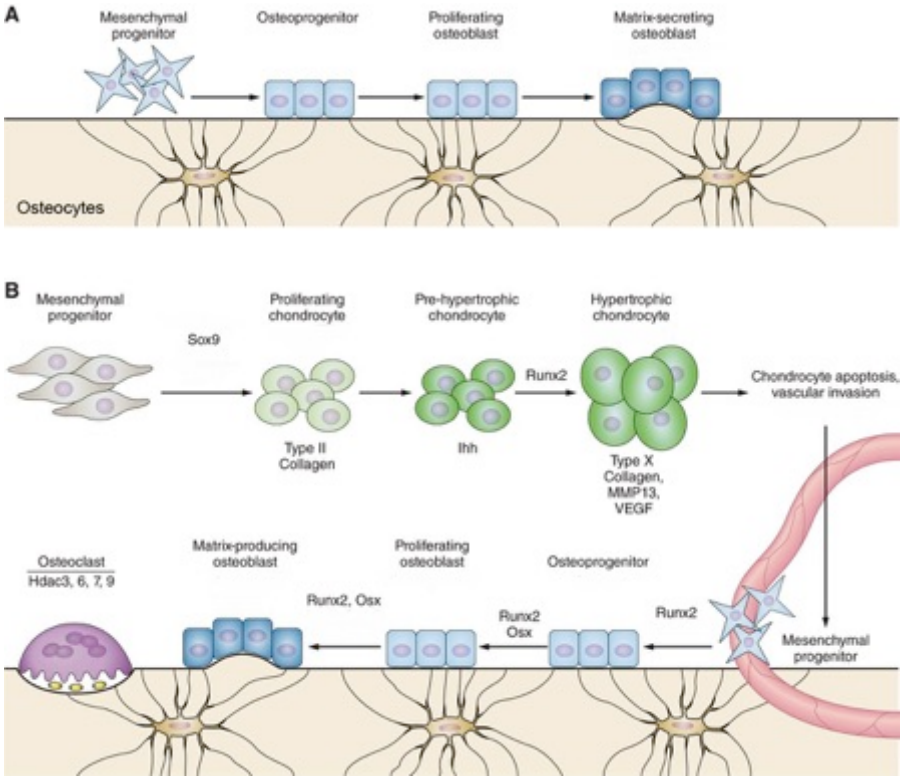

Figure 6: Bone Formation
Two mechanisms produce bone. **A.** Intramembranous ossification occurs principally during foetal development when mesenchymal progenitors differentiate directly into osteoblasts. **B.** Endochondral ossification produces bone after birth and during fracture healing; it requires a cartilage step. Adapted from Uccelli *et al.* (2008) [36].

During endochondral ossification (Figure 6B), mesenchymal progenitors condense; this is the signal for MSCs to commence differentiation. The first step leads to proliferating chondrocytes, which form hyaline cartilage with the concurrent expression of high levels of type II collagen, proteoglycans and SOX9. Once fully differentiated, chondrocytes stop proliferating and undergo hypertrophy characterised by a reversal from type II to type X collagen [62]. The hypertrophic cartilage is then resorbed and invaded by osteoblast precursors. This process initiates the formation of bone as well as hematopoietic and

endothelial cell components for bone-marrow establishment [63]. This process has been identified as the primary ossification centre, which expands with body growth to form long bones. At the epiphyses, another site of bone production called the “secondary ossification” centre is initiated; it is the origin of epiphyseal growth-plate cartilage (Figure 7).

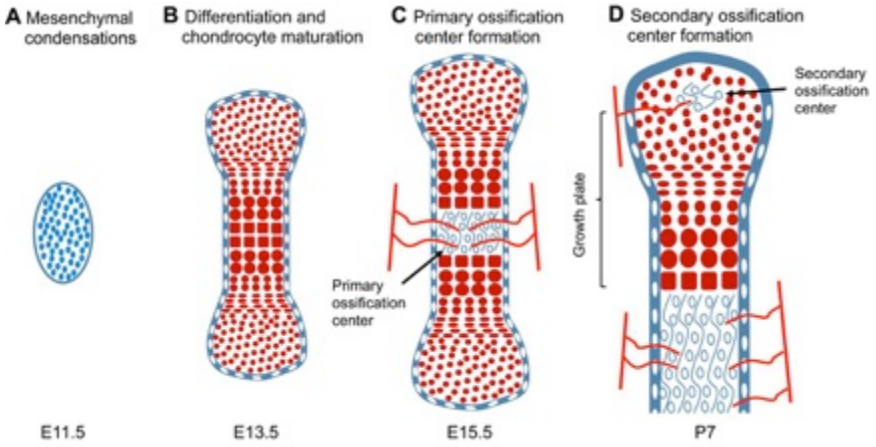

Figure 7: Stages of Endochondral Ossification in the Long Bones of Mice
A. Mesenchymal progenitors (blue cells) condense to initiate a differentiation process at 11.5 days of embryonic development (E11.5). B. Within two days, MSCs have already become chondrocytes (red cells) and start entering hypertrophy. Immature cartilage is protected by the perichondrium (white cells). C. In the establishment of the primary ossification centre at E15.5, vessels (red lines) start invading the area of hypertrophic chondrocytes, allowing calcification of the cartilage (blue zone). D. Seven days after birth (P7), the secondary ossification centre (blue zone) is formed at the epiphyses and vascularised (red line). This process is the origin of the epiphyseal growth plate [62].

1.3.2. Bone Cells

Bone sustainability and homeostasis are based on the strictly regulated activities of osteoblasts, bone lining cells (BLCs), osteocytes and osteoclasts [64,65].

Osteoblasts represent the bone-forming cell population resulting from MSC differentiation. They are organised on the bone surface and exhibit a cuboidal phenotype. Osteoblasts’ polarisation is decisive in the synthesis and secretion of protein and vesicles in the bone matrix [66].

BLCs are flat, quiescent osteoblasts that cover the bone surface at sites where no bone remodelling occurs. However, under the influence of parathyroid hormone (PTH), FGF-2 or mechanical loading, BLCs have the ability to acquire a cuboidal shape and activate their secretory system. As a result, they can ensure the osteoblast pool when bone disruption occurs [67]. Bone lining cells affect cell density and play a significant role in controlling the process of bone remodelling [68,69].

Osteocytes are the final stage of MSC differentiation during bone formation. Their complete differentiation requires osteoblasts to enter four successive phases: (i) osteoid-osteocyte, (ii) pre-osteocyte, (iii) young osteocyte and (iv) mature osteocyte embodied in the lacunae [70]. Due to their location within the matrix, osteocytes have the ability to sense mechanical stress. As a result, they regulate their actin cytoskeleton dynamics to produce a biochemical signal in response to the matrix environment. Bone tissue can integrate and respond to this signal and subsequently adapt to daily physical forces [71]. Osteocytes also have the capacity to regulate bone-remodelling events indirectly through their influence on osteoclasts and osteoblasts. Indeed, osteoclasts can be activated following osteocyte apoptosis [72,73].

Osteoclasts originate from the hematopoietic cell lineage, and their differentiation is orchestrated by a variety of factors. Osteoprogenitors and osteoblasts secrete macrophage colony-stimulating factor (M-CSF), which binds to receptors on osteoclast precursors, activating cell proliferation and inhibiting apoptosis [74-76]. Osteoblasts, osteocytes and stromal cells produce the receptor activator for nuclear factor κ-B ligand (RANKL), which binds to its receptor RANK present on osteoclast precursors and induces osteoclastogenesis [77,78].

1.3.3. Bone Function

Despite the skeleton being generally viewed as an inert structure it is a well vascularised and dynamic organ. Bone tissue is tightly regulated and shows a high level of organisation in order to permit structural support and locomotion.

Furthermore, the skeleton is responsible not only for protecting and regulating inner organs which can in turn communicate to the bone tissue in a feedback loop and influence its activity [79].

Bone tissue acts as a calcium and phosphate reservoir to prevent compromising concentrations that may have a negative influence on the human body as a whole (i.e. blood pressure) [80]. Calcium is released in response to stimulation and can be used for bone formation or as a second messenger for signal transduction. Bone can store not only physiologic ions but also toxic compounds when exposure is too high [81]. Moreover, bone plays an important role in fat storage, which can be used as a source of energy in the differentiation of HSCs [82].

Another function for bone tissue is its involvement in pH homeostasis due to its ability to absorb alkaline salts responsible for pH variations.
Additionally, bone tissue exhibits endocrine properties by releasing hormones and growth factors. For example, it is known that osteocalcin (OCN/BGLAP) participates in insulin regulation, as well as FGF-23 controls phosphate homeostasis [83]. Finally, the bone marrow, source of BMSCs, is embedded within the bone cavity, [84].

1.3.4. Bone Turnover and Remodelling

Bone turnover is essential for bone formation and repair (i.e. after fracture). It enables mineralized bone to respond to biomechanical stress and regulates the body's calcium supply [79]. Bone turnover is a highly regulated process involving the removal of old bone by osteoclasts and the formation of new bone by osteoblasts [85-87]. The strict control of bone remodelling is due to the activity of various signals such as hormones, cytokines, chemokines and biomechanical stimulation.
The process of bone remodelling consists of osteocytes transforming biomechanical stress into a biochemical signal. Although the process is still not completely understood, some hypothetical mechanisms of action for bone remodelling have been suggested:

- First, a trans-membrane protein called polycystin-1 (PC1) belonging to the mechano-sensor complex polycystin-2 (PC2) is expressed by osteoblasts and osteocytes. It was demonstrated that homozygous and heterozygous mutant mice for *Pkd1*, the gene encoding for PC1, exhibited delayed endochondral and intramembranous ossification or osteopenia due to a significant decrease in osteoblastic activity. Moreover, loss of PC1 function is associated with a down-regulation of *Runx2* gene expression, while PC1 constitutive overexpression results in an increase of *Runx2* and the gene expression of osteogenic markers [88,89]. These observations suggest that PC1 could possibly represent a fundamental key player in the initiation of bone remodelling.
- The second possible mechanism relies on the focal adhesions of osteocytes, which may be the effectors of osteocytes' secondary messengers, such as adenosine triphosphate (ATP), nitric oxide (NO),

calcium and prostaglandins. Loss of osteocyte adherence could trigger the activation of osteoclasts. Therefore, the modulation of the actin cytoskeleton participates in the regulation of bone morphology [90-93].

The equilibrium between bone formation and bone resorption relies on communication between bone components. Osteoblasts and stromal cells can inhibit osteoclast proliferation and differentiation by secreting osteoprotegrin (OPG). OPG binds and sequesters RANKL to prevent its interaction with RANK (Figure 8). If the RANKL/RANK axis is not activated, the nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), will not activate tartrate-resistant acid phosphatase and cathepsin K gene expression, which is required for osteoclast activity [60,94-96].

During bone remodelling, active osteoclasts are precisely oriented along the bone matrix. The sealing and ruffled zones are both in contact with the matrix; the situation is different with the baso-lateral and secretory zones. This orientation is decisive for osteoclasts to efficiently degrade the bone matrix, and it involves cytoskeleton rearrangements [97-99] (Figure 8). Osteoclasts form podosomes in the ruffled zone as a result of the formation of an F-actin ring. This process occurs in order to maintain the ruffled zone and lacuna as isolated sites where bone resorption takes place [100,101]. The ruffled zone is an area of heavy molecular traffic, and the lacuna is an acidic environment. Furthermore, maintenance of low pH is crucial for a successful bone resorption and is ensured by a vacuolar-type H^+-ATPase (V-ATPase) [102]. In the lacuna, osteoclasts secrete protons, TRAP, cathepsin K and matrix metalloproteinase-9 (MMP-9). These components are active at an acidic pH and degrade hydroxyapatite (HA) crystals [103,104].

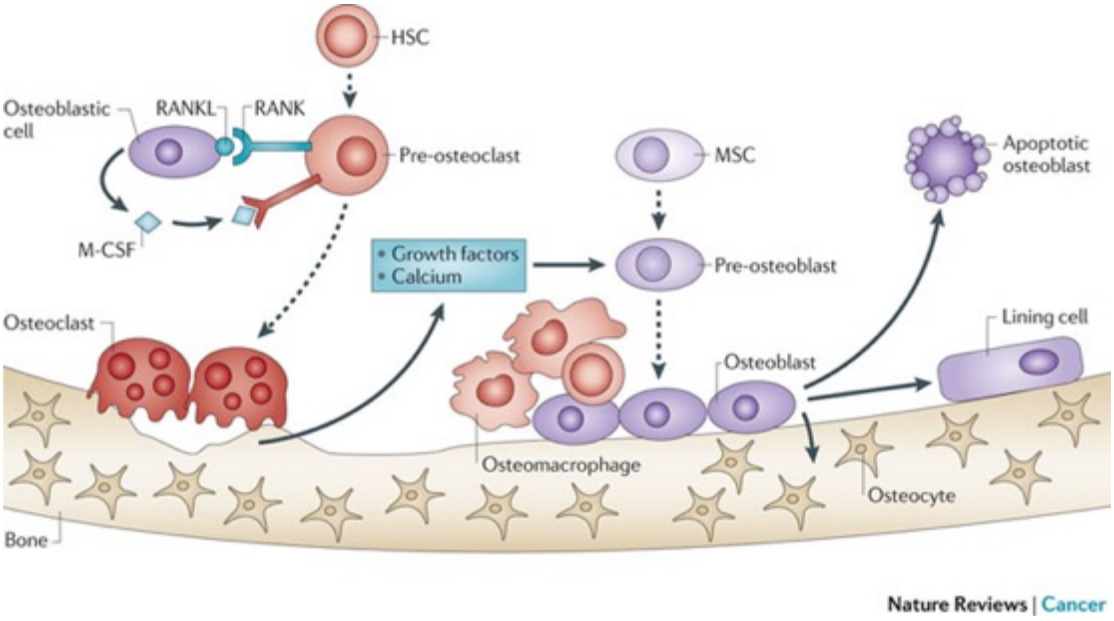

Figure 8: Bone Remodelling
Originating in MSCs, osteoblasts regulate bone production, while osteoclasts, which belong to the hematopoietic lineage, have resorption properties (dashed arrows). The bone matrix is replaced in a tightly regulated feedback loop (solid arrows) [105].

1.4. Molecular Regulation of Osteogenesis

Osteoblasts are MSC-derived cells, and their differentiation depends on several successive and closely regulated phases (described in 1.4.1) in which pathways involving Wnt and BMPs play a major role (detailed in 1.4.2) [106].

1.4.1. Signalling Events

Osteoblast differentiation can only take place if the following three genes are expressed: runt-related transcription factor (*RUNX2*), distal-less homeobox 5 (*DLX5*) and osterix (*OSX*) [107].
These genes initiate a cascade of events that lead to mature osteoblasts. First, activation of RUNX2 protein triggers the transcription of osteogenic markers such as collagen type I A1 (*COL1A1*) and alkaline phosphatase (*ALP*) [108]. Subsequently, osteoblast progenitors expressing *ALP* enter a proliferative phase; at this point, they can be referred to as pre-osteoblasts [109]. The final switch to mature osteoblasts is ensured by the expression of *OSX* and *OCN*. Eventually, mineralization of the bone matrix occurs. Osteoblasts secrete matrix vesicles at their apical domain within the unmineralized bone. The vesicles are filled with

calcium ions and can be captured by inorganic and negatively charged components such as proteoglycans. Proteoglycan degradation by osteoblast-secreted enzymes enables the release of calcium ions through annexins (calcium channels) in the vesicle's membrane [66,110]. Meanwhile, osteoblast-secreted ALP dephosphorylates a diverse range of components to generate a pool of free phosphate ions. These phosphate ions are then stored in the matrix vesicles, where they fuse with calcium ions to form HA crystals. This fusion is the vesicular phase, and it is followed by the fibrillar phase, which leads to the breakdown of the vesicle membrane due to saturation by calcium and phosphate. Hydroxyapatite crystals are released and bone tissue is formed through mineralization of the matrix. Three different outcomes are now possible for osteoblasts: (i) entering apoptosis, (ii) differentiating into osteocytes or (iii) differentiating into BLCs [26,111]. In the final stage of differentiation, the expression of osteoblast markers (e.g. *OCN*, *COL1A2*, *ALP*) is down-regulated in favour of osteocyte marker expression (e.g. dentine matrix protein 1 (*DMP1*), sclerostin (*SOST*), and podoplanin (*E11*)) [112,113].

1.4.2. Cross-talk Between Signalling Pathways Promotes Osteogenesis

Mesenchymal stem cell differentiation into osteoblasts is subject to tight regulation involving several pathways. Osteogenesis can be enhanced by the combination of different pathways promoting osteoblast maturation.

Hedgehog signalling plays a crucial role in skeleton formation during development [114]. Indeed, Shh can promote osteogenic differentiation by increasing TGF-β2 levels, which lead to the inhibition of chondrogenic lineage in favour of osteogenic lineage. Moreover, Shh and Gli2 can synergistically trigger *BMP2* expression, a key pillar for bone formation [115]. Additionally, Indian hedgehog (Ihh) together with Gli2 stimulates the expression and transcription activity of *RUNX2* [116].

Wnt signalling acts via several effectors implicated in cell renewal, osteoblast differentiation and inhibition of osteoblasts apoptosis to produce and maintain bone [117]. BMP-2 activates Wnt signalling pathways by increasing the production of Wnt ligand, its receptor Frizzled and co-receptor LRP5/6 [27]. Moreover, production of the WNT10B protein favours the expression of *Runx2*, *Dlx5* and *Osx*, key factors of osteogenesis and inhibition of adipogenesis [118]. Additionally, activation of the Wnt canonical pathway stabilizes β-catenin and

prevents its degradation. β-catenin is then able to translocate to the nucleus, where it regulates gene transcription [119,120]. Furthermore, β-catenin, together with RUNX2, promotes transcription of osteocalcin during BMP-9-mediated osteoblast differentiation [120]. Finally, RUNX2 also associates with transcription factor 4 (TCF4), and this complex leads to an increase in TGF-βR1 transcription, triggering osteogenesis [121].

Mitogen-activated protein kinase (MAPK) pathway regulates osteogenic lineage commitment through extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) and p38. Preventing MAPK pathway to signal via its osteogenic effectors promotes adipogenic differentiation [122].

Micro-RNAs (miRNAs) have recently attracted interest due to their ability to regulate MSC differentiation [123,124]. Some miRNAs have been found to have a stimulatory effect on the differentiation of osteoblasts, such as miR-218 and miR-2861, by repressing *SOST* and histone deacetylase 5 (*HDAC5*) respectively. Conversely, other miRNAs have been found to have a detrimental effect on osteogenesis by targeting *RUNX2* gene expression [125,126].

The Notch pathway is involved in a plethora of biological processes, and its involvement in bone formation by stimulating the BMP/SMAD axis has already been demonstrated [127]. The notch intracellular domain interacts with transcription factors to modulate *RUNX2* gene expression. Additionally, Notch can influence the balance between osteoblast and osteoclast activity by regulating the ratio of RANKL/OPG [128].

1.5 Fracture Repair

Trauma, tumour resection, reconstructive surgery, congenital malformations and infections are some of the reasons a bone may fracture or be sectioned during a person's lifetime [129]. The regenerating process to form new bone tissue that cannot be discerned from existing bone is based on direct remodelling (intramembranous ossification) and indirect remodelling with cartilage callus formation (endochondral ossification) [130].

Reconstruction of the injured bone occurs during a three-phase process: inflammation, renewal, and remodelling. Indeed, after bone fracture, pro-inflammatory signals initiate the inflammatory response essential to recruit factors needed for new bone formation and bridging the fracture gap [131].

The trauma generated by bone disruption unleashes pro-inflammatory signals and growth factors to recruit tumour necrosis factor-α (TNF-α) and interleukins such as IL-1, IL-6, IL-11 and IL-18 at the fracture gap site [132-135]. As a consequence of chemokine accumulation, neutrophils and macrophages migrate to the injured area in order to clear the wound by endocytosis of debris produced by the fracture [132,136]. Furthermore, bone is a vascularized organ, and the rupture of blood vessels results in platelets producing platelet-derived growth factor (PDGF) and TGF-β1. These early steps of inflammatory response promote the formation of a hematoma composed of macrophages that secrete TGF-β, insulin-like growth factors (IGFs) and FGF-2. Along with the inflammation process, the neighbouring osteoprogenitors express BMPs [137]. The combination of factors and cascade of events recruits MSCs, thereby initiating the renewal phase [138].

During the renewal phase, which occurs 7 to 10 days after injury in the periphery of the injured tissue, MSCs proliferate and undergo osteogenesis via intramembranous ossification [137,138]. The next step is the formation of a cartilaginous callus within the injured tissue through initiation of chondrogenesis; this step marks the end of the inflammatory phase [35,133]. Endochondral ossification is the main mechanism that replaces cartilage with bone at fracture sites, and is under the control of BMPs, TGF-β2 and TGF-β3 [139,140].

Reconstruction of the newly formed bone into lamellar bone results from the interplay between osteoblast and osteoclasts, and constitutes the remodelling phase [137]. Bone formation and resorption are under the tight regulation of inflammatory factors such as IL-1, IL-6, TNF-α and interferon gamma (IFNγ) (Figure 9) [140].

Skeletal development and fracture healing share similar features with respect to signalling pathways involved in MSC osteogenesis, even though inflammation does not take place during skeleton development and fracture healing does not involve ESCs [137].

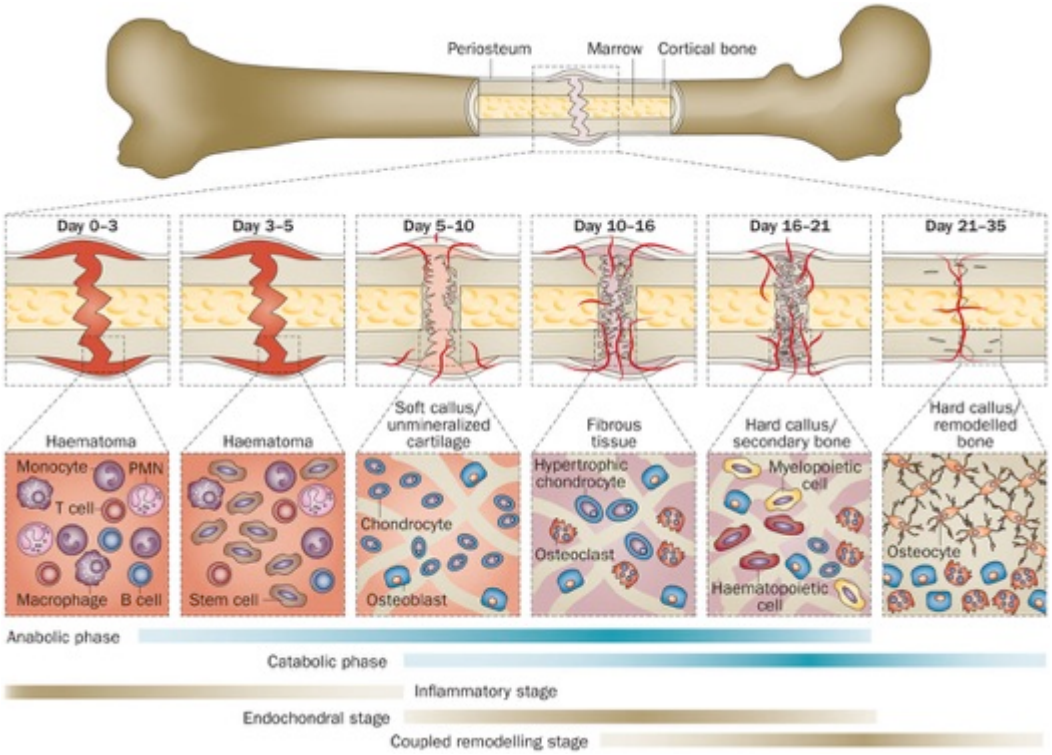

Figure 9: Bone Fracture Healing Process in Mouse Femur
The primary metabolic phases occurring during bone fracture healing leans on the three biological stages: inflammation, renewal (endochondral bone formation) and restructuration (remodelling) [119].

Despite bone's remarkable capacity to regenerate and repair, sometimes an injury is too severe, or healing conditions are not optimal (infection, insufficient blood supply) for efficient recovery. In such cases, clinical intervention is required to promote bone repair [129]. To date, the most widely adopted technique to treat critical bone defects is the autologous bone graft, which consists of transplanting a patient's cancellous or cortical bone tissue from one part of the body to the injured site [141-143]. Allografts are bone tissues obtained from an organism and transplanted into an individual of the same species. They are also routinely used as an alternative to autologous grafts.

Fresh grafts express osteogenic factors such as BMP-2, FGF, IGF and PDGF, and cells can survive for a short period of time depending on the graft's type [144].

For successful bone generation, a graft should exhibit specific traits:

- Osteogenesis: the graft allows osteoblast differentiation [145,146].
- Osteoinduction: the graft material is able to promote the formation of bone-forming cells [147].

- Osteoconduction: the graft material provides a scaffold to the newly formed bone tissue that can be maintained after the resorption of the graft transplant [148].
- Osteointegration: the newly formed bone must connect with the already existing bone tissue without the integration of fibrous layers [149].

Nevertheless, patients and clinicians still face constraints such as the availability of compatible donor tissue, second surgeries to collect graft tissue from a patient, and serious post-operative complications. Hence, the development of alternative methods to overcome therapeutic limitations relies on a good basic understanding of the mechanisms controlling MSC differentiation [150].

1.6. The Role of MSCs in Bone Repair and Disease

Bone quality is highly dependent on the functional activity of its resident MSC population, and any alteration in the differentiation pathway of these cells may result in altered lineage commitment that in turn may lead to bone disorders. MSCs initially attracted interest due to their multipotency, as well as their potential use in stem-cell therapy. Additionally, their ability to differentiate into various tissues (*in vitro* and *in vivo*), such as adipose tissue [151], tendons [74], muscle, cartilage and bone [152], renders them ideal candidates for regenerative medical approaches.

1.6.1. Osteoporosis

Osteoporosis (OP) is characterized by a reduction in bone mass and degradation of bone's microarchitectural structure [153]. It is a systemic disease affecting the whole skeleton and results in loss of mechanical strength, decrease in bone mineral density (BMD) and increased fracture risk [154]. Osteoporotic bone loss is due to an anarchic bone degradation associated with excessive osteoclast activity and impaired MSC commitment favouring adipogenesis over osteogenesis [155]. Numerous risk factors (genetic, environment and physical activity) and haplotypes with pleiotropic mutations, such as vitamin D receptor (*VDR*), *COL1α1*, oestrogen receptor 1 (*ESR1*) and Wnt signalling, have been identified. Despite these findings, no particular mutation setting is independently responsible for OP.

OP can be classified as primary (itself subdivided into two distinct types) or secondary.

Type I primary OP or post-menopausal osteoporosis is the most common form of bone loss in women due to the cessation of oestrogen production. Patients suffering from type I OP exhibit mainly trabecular bone deteriorations such as low trabecular bone mass and disruption of the trabecular bone architecture. Cortical bone is also affected by the bone resorption, which expands the modularly cavity and leads to a reduction in cortical bone mass. The severe weakening of the bone in type I OP predominantly predisposes women to vertebral and wrist fractures [156].

Type II primary OP, or senile OP, is related to aging, which results in hormone variations in women and men [157] that can progressively contribute to bone loss affecting several sites of the human skeleton.

Secondary OP [158] is a particular case in which the degradation of the bone is mainly due to the use of glucocorticoid (GC) in the management of pain in musculoskeletal and rheumatoid disorders or herniated disk [159]. Chronic exposure to GC is responsible for 20% of secondary OP cases [160] by destabilisation of the equilibrium between bone resorption and bone formation [161]. GC treatment impairs MSCs differentiation into osteoblast [162], promotes their adipogenic fate [163] and stimulates osteoclast activity due to increases in RANKL production [164]. Secondary OP can also results from other pathological conditions such as hypogonadism, renal failure, rheumatic disease, osteomalacia, endocrine and gastrointestinal deregulations, rickets and Paget's disorders.

A considerable amount of evidence now exists from studies using experimental animal models to support a role for dysfunctional MSC differentiation in determining bone quality [165].

1.6.1.1 Animal models of age-related bone loss

SAMP6 mice

The senescence-accelerated mouse prone 6 (SAMP6) [166] model exhibits a phenotype comparable to human osteoporosis with spontaneous bone fractures [167,168]. It was shown that BMSCs originating from SAMP6 mice preferentially differentiated into adipocytes rather than osteoblasts [169]. However, the precise

cause for reduced bone mass in SAMP6 mice remains unclear. It has been suggested that the impaired bone phenotype is due to a mutation in the gene encoding interleukine-4 receptor (*Il4ra*), resulting in osteoclast over-activation and subsequent increased bone loss [165]. However, it was also shown that mice overexpressing *Il4* exhibited a decrease in bone formation and quality [170].

Klotho deficient mice

The Klotho mouse model carries a disruption of a type-I membrane protein [171]. The Klotho deficiency is accompanied by an increase in renal activity resulting in hyperphosphatemia, which was also linked to premature aging [172]. Mice homozygous for the Klotho mutation displayed various aging phenotypes including reduction in cortical bone thickness explained by lower values in bone formation parameters such as osteoblast surface per bone surface and bone formation rate per surface. In contrast, trabecular volume and thickness were increased [173]. In this mouse model, osteogenesis of BMSCs was also impaired, being directly related to deficiencies in the Klotho protein, which is thought to regulate fibroblasts growth factor 23 (FGF23), a known inhibitor of bone mineralization [174]. Moreover, Klotho-deficient BMSCs preferentially differentiated toward adipocytes [173,174].

Telomerase deficient mice

Maintenance of telomere length is crucial to prevent aging and is ensured by telomerase, which is composed of a reverse transcriptase subunit (TERT) as well as an RNA component (TERC). It was previously described that functional deficiencies in TERC prevent telomere stability, accelerate aging and decrease BMD in mice [175,176]. Furthermore, isolated BMSCs from *Terc*$^{-/-}$ mice demonstrated a decreased potential to differentiate into osteoblasts [175,177]. Interestingly, telomere length was also compromised in SAMP6 mice, thereby further supporting the theory that telomere shortening may have an influence on bone loss [178]. Additionally, mice deficient in the Werner helicase (Wrn) also develop an accelerated aging phenotype. *In vitro* experiments using BMSCs isolated from *Wrn*$^{-/-}$ and *Terc*$^{-/-}$/*Wrn*$^{-/-}$ mice demonstrated significant reductions in their ability to undergo osteogenesis. Furthermore, significant increases in fat

marrow content were also observed in *Wrn*$^{-/-}$ and *Terc*$^{-/-}$ *Wrn*$^{-/-}$ mice as compared to wild-type controls [176,179].

Mouse models of transcription and DNA repair disruption

The trichothiodystrophy (TTD) mice carry a mutation in the xeroderma pigmentosum factor D (XPD) helicase, and display impaired cortical bone formation in association with deficiencies in BMSC osteogenesis [180,181]. Similarly, mice partially or completely deficient in excision repair cross-complementary group 1-xeroderma pigmentosum group F exhibit an osteoporotic phenotype, along with deficiencies in BMSC osteogenesis [182]. In addition, it was also observed that disruptions in the Cdc42 GTPase activating protein (Cdc42GAP) have a negative impact on mouse bone microarchitecture [183]. Furthermore, Cdc42GAP deficiencies were associated with over-production of p53. Indeed, up-regulation of *p53* is a pattern that was also found in Hutchinson-Gilford progeria syndrome (HGPS) [184] as well as in *Zmpste24*-knockout (a metalloproteinase) mice [185], which exhibit severe reductions in both BMD and BMSC osteogenesis. These results therefore suggest that DNA repair plays a decisive role in BMSCs commitment and subsequent bone formation. They also imply that p53, despite it being considered to be a pro-survival element in response to cell stress, may have a detrimental effect on age-related bone loss [186-188].

In order to supplement the results gathered by the previous mice models, numerous studies have investigated the osteogenic potential of human BMSCs (hBMSCs). It was reported that hBMSCs isolated from osteoporotic patients exhibited a decreased potential in matrix mineralization [189,190] as well preferentially differentiating into adipocytes due to the type I collagen deficiency of the ECM [191]. Furthermore, osteoblastogenesis impairments of hBMSCs isolated from osteoporotic patients could be rescued after treatment of the cultures with aminobisphosphonate [192]. These results imply that targeting stem cells may be an alternative approach to treating age-related bone loss.

1.6.1.2 Use of MSCs to treat bone loss

Based on the fact that numerous age-related osteoporosis-like mouse models display abnormalities in BMSC lineage commitment, therapies targeting stem cells may therefore represent a promising approach to treat bone loss. Indeed, studies have already demonstrated that isolation of the bone marrow cells from C57BL/6 mice and subsequent injection into osteoporotic SAMP6 mice could restore trabecular structure [193,194]. Furthermore, it was shown that bone quality as well as osteoblast number were significantly improved in the *Wrn*$^{-/-}$ *Terc*$^{-/-}$ mice following bone marrow transplantation from young wild-type donors, diminishing the effect of accelerated aging on bone structure [195]. It is important to note that irradiation and transplantation of the complete BM were required to induce significant improvements in bone parameters of the above mice. Additionally, BM transplantation is an invasive technic with challenging immunologic issues. Therefore, researchers and clinicians started to consider other sources of MSCs for bone loss treatment.

Based on the osteogenic deficiencies associated with BMSCs from osteoporotic and aged patients, there's a growing interest in identifying other alternative MSC sources. Several groups have already demonstrated that MSCs isolated from adipose tissue, termed adipose-derived stromal cells (ASCs), can prevent bone loss occurring in ovariectomized (OVX) mouse models [196-198]. Moreover, ASCs isolated from male SAMP6 mice were shown to improve bone quality when re-injected into female SAMP6 recipients [199]. Similarly, ASCs isolated from human osteoporotic patients were able to successfully commit toward osteogenesis [178,190], whilst the osteogenic capacity of the corresponding BMSCs remained significantly impaired [189,190].

The observation that osteogenic differentiation of ASCs from aged donors remains unimpeded, may be explained by differences in tissues methylation, resulting in altered rates of aging [200]. Several studies have already shown that ASCs represent promising candidates to treat bone loss and are now used for autologous cell-based therapies for orthopaedic tissue repair [201,202].

1.6.2 Fracture repair

Although considered as the gold-standard procedure, autologous bone grafts display several limitations such as morbidity at donor site [203] and low bone quality in the case of OP [189,203]. Recent clinical studies have evaluated the use of artificial scaffolds to promote osteoprogenitor differentiation and bone healing [204-206]. Despite the positive effect of this combination to treat injured bone, the transplanted cells did not exhibit a particular role in enhancing bone regeneration and the costs for such a procedure remain too high to use as a standard intervention [207]. It is therefore of common interest to find alternative solutions to optimize bone regeneration. The growing interest in the use of autologous MSCs is exemplified by studies in which MSCs have been used to promote the healing of certain bone injuries [85,86].

The stromal vascular fraction (SVF) of the adipose tissue is composed of MSCs (ASCs) able to differentiate into osteoblasts as well as endothelial cells able to establish a vascularization of the tissue [208,209]. The SVF can be harvested by non-invasive technics, is easily accessible, and provides considerable amounts of autologous osteoprogenitors. The isolated cells can be seeded and implanted within approximately 4h [210], which considerably speeds up the procedure as compared to the 6 weeks normally needed for the traditional autologous bone graft approach [211]. It was previously found that SVF grafts promoted bone formation only under osteogenic stimulation thus, avoiding ectopic bone formation [212].

The scope of the clinical evaluation using stromal cells to improve bone healing is rather small. Despite numerous disparities in cell source, technics, the heterogeneity of the patients as well as regulatory issues, few studies reported the beneficial effect of stromal cells. Indeed, ASCs were used in the largest experimental protocol established to treat cranio-maxillofacial hard-tissues defects [213]. Despite the positive clinical outcomes, the exact role for ASCs in these grafts remains unclear due to non-standardised factors such as the use of different types of scaffolds, treatment with exogenous BMP-2 and the severity of the patient's injury. Additionally, the cells used for the graft probably did not display all their multipotent capacities since they were harvested and cultured *in vitro* prior to the transplantation, which impairs their potential to differentiate [214].

To date, the use of freshly isolated adipose-derived cells to treat critical bone defects have been reported in three independent cases of hip necrosis treatment or cranial reconstruction [215-217]. However, even though the cells were not expanded *in vitro*, the authors used stimulating factors to induce their differentiation.

Saxer *et al.* (2016) [210] demonstrated for the first time that the implantation of human SVF into a critical-size defect of femur in the rat could form new bone tissue and restore vascularization without any prior or exogenous stimulation. This approach was further extended to a first-in-man clinical trial to treat humeral fracture due to OP where *de novo* bone formation was identified at the site of implantation [210]. It is believed that the underlying mechanism triggering SVF cells to an osteoblastic fate relies on the inducible osteogenic capacities of the ASCs stimulated by the surrounding environment [218]. Hence, the SVF is considered as an appropriate source to supply the required factors to improve bone healing [219] since the *ex vivo* expansion of the cells is no longer needed which solves regulatory and biological issues [220].

However, the mechanisms governing SVF osteogenic differentiation remain controversial. For instance, it's still unclear whether SVF cells are dependent on exogenous human BMP-2 stimulation [220], and whether they require an *in vitro* expansion step [214] or can be used as a primary cell population [210].

Together, these results have established MSCs as a powerful tool for tissue regeneration and cell therapy. Nonetheless, the need to optimise and establish a lasting and optimized solution is becoming evermore important in a society in which people expect and are expected to live longer.

1.7. HTRA1

High temperature requirement A (HtrA) proteases are well-conserved proteins originally identified in bacteria [221] (Figure 10). In mammals, the HtrA family is composed of four members: HTRA1, 2, 3 and 4. Mammalian HtrA family members are implicated in numerous biological processes such as cell death, signalling, development and motility. Because they act in multiple cell functions, HtrA impairment plays an important role in many disorders. As such, these proteases are considered potential therapeutic targets [222,223].

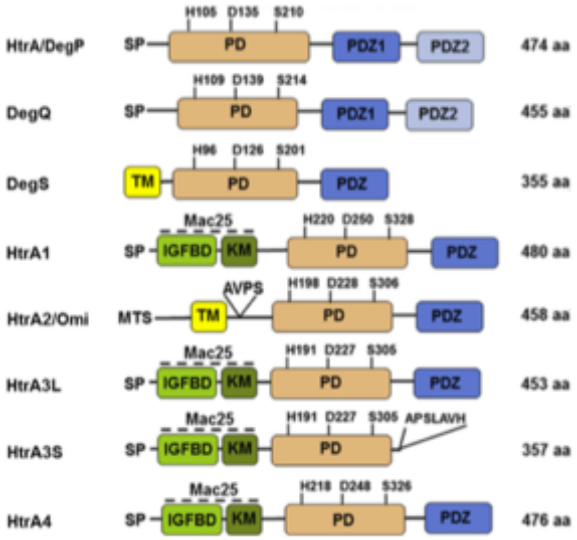

Figure 10: Structural Comparison of Bacterial and Human HtrA Proteases
AVPS: interaction with IAPs; AVPSLAVH: replaces PDZ in HTRA3 short form; IGFBP: high homology with insulin-like growth factor binding protein; KM: Kasal-type serine protease inhibitor motif; Mac25: high homology with Mac25 protein; PD: chymotrypsin-like protease domain with amino acid catalytic triad H-D-S for Histidine – Aspartate – Serine; PDZ: PDZ domain; TM: transmembrane domain; SP: signal peptide [224].

In the following section, I primarily focus on the first member of the HtrA family, HTRA1, which is a secreted protease expressed ubiquitously in practically every tissue [225]. I provide a detailed description of HTRA1's structure, biological functions and involvement in several pathologies, and address several grey areas concerning HTRA1's mode of action.

1.7.1. Structure

Structurally, HTRA1 carries five different domains (Figure 10) and shares a similar amino acid sequence profile with its paralogs HTRA3 [226] and HTRA4, suggesting possible overlapping roles between these members [227]. Furthermore, in mice and human HTRA1 and HTRA3 have been identified at similar locations [227,228].

1.7.1.1. Signal Sequence

HTRA1, HTRA3 and HTRA4 contain a signal peptide (SP) at their N-terminal domain that contributes to the initiation of their secretion [221]. Despite the crucial role of the SP domain in HTRA1 cell transport, the underlying mechanism in charge of the secretion remains unknown [229].

1.7.1.2. IGFBP/Mac25 Domain

The signal peptide domain is structurally followed by the Mac25 domain, which shares features with the insulin-like growth factor binding protein domain (IGFBP). It is believed that the Mac25 domain has an unconventional role, which differs from the original purpose of the IGFBP to interact with IGFs, since no interaction has been identified so far. In addition to the similarities shared with IGFBP, Mac25 also displays high homologies with the Mac25 protein, a follistatin-like protein, but does not have the same functions [229] and does not affect HTRA1 proteolytic activity either [230,231]. One proposed role of the Mac25 domain was in substrate recognition and triggering proteolysis to help with binding and cleavage of IGFBPs to subsequently modulate IGF signalling [232]. Additionally, it was found that the TGF-β pathway can be altered by HTRA1, and is dependent on HTRA1's proteolytic activity as well the presence of the Mac25 domain [233]. However, a deeper understanding of this domain in HTRA1 substrate specificity is still needed.

1.7.1.3. Kasal-type Inhibitory Domain

The Kasal-type serine protease inhibitor motif (KM) is located upstream of the protease domain. The KM domain is known to represent the inhibitory sequence of serine proteases due to its capacity to bind their proteolytic sites and inhibit their interaction with the targeted substrate. Even though HTRA1's KM domain has not yet been shown to exhibit any inhibitory effect, it has been suggested that the KM domain inhibits HTRA1's protease domain in the absence of substrate, thereby preventing premature activation of HTRA1 [231,234,235]. Moreover, it is common to find the IGFBP/Mac25 domain associated with the KM domain in numerous proteases; this situation implies a specific role for this combination, but the precise function has yet to be determined [236].

1.7.1.4. Protease Domain

HtrA serine proteases share a particular feature: a trypsin-like protease domain (PD) [236] responsible for cleaving a peptide bond in the targeted protein

following a positively charged amino acid. The protease domain targets various substrates, and substrate specificity depends on the following:

- The triple amino-acid pattern of the catalytic triad Histidine, Aspartate and Serine regulating HtrA's catalytic activity [237].
- The secondary structure of the proteolytic pocket.
- Other domains carried by the protease [238].

The catalytic triad is a well-conserved motif responsible for proteolytic activity. More specifically, a serine residue drives the nucleophilic attack of the carbonyl group on the targeted peptide bond. The disruption of the covalent bond breaks the protein down into two final peptides. Hence, HTRA1 can also be labelled as an endopeptidase [239]. In bacteria, it has been demonstrated that the association of HTRA1's PD domains in barrel-like structures can enhance the protease's enzymatic activity. These structures are thought to increase HTRA1's chaperone and proteolytic activities [56,230,240].

To date, two different models of activation for HTRA1 have been proposed:

- The substrate-induced model suggests an activation of HTRA1 *via* the interaction between the target and the PD. Conformational changes induced by the interaction between the substrate and the PD could be responsible for HTRA1 activation [230].
- The conformational selection model [229] hypothesises the possible co-existence of HTRA1 in active and inactive forms, even in the absence of a ligand. It is thought that ligand binding would destabilise the equilibrium between active and inactive forms of HTRA1, thereby inducing protease activity of the catalytic complex.

1.7.1.5. PDZ Domain

Localising at HTRA1's C-terminal region, the PDZ domain is the repetition of motifs found in three different proteins: postsynaptic density of 95 kDa (PSD-95), drosophila discs large tumour suppressor (DLG) and zonula occludens 1 (ZO-1).

The HTRA1 PDZ domain is involved in the regulation of HTRA1 activity by binding to substrates and regulatory peptides and mediating HTRA1 interaction with membrane structures. The PDZ domain is highly conserved, provides substrate specificity and can be found in several proteases [241,242]. The interaction between the PDZ domain and the substrate is a complex process

involving several biological responses [243]. In the case of HTRA1, after interaction with the substrate, the PDZ domain induces conformational changes of the PD domain that lead to an enhanced catalytic activity by suppressing steric hindrance [229,244]. However, other studies found that deletion of the PDZ domain did not affect protease activity [230].

Furthermore, HTRA1 exists as a single stable and soluble molecule that can produce higher complexes to adapt to different environmental conditions [230]. Indeed, HTRA1 was demonstrated to assemble into trimers stabilized by PDZ-PDZ interactions (Figure 11). It is conceivable that the oligomerization of HTRA1 is a key regulator of the active and inactive states of the protease, with the resting form being the homotrimer [245].

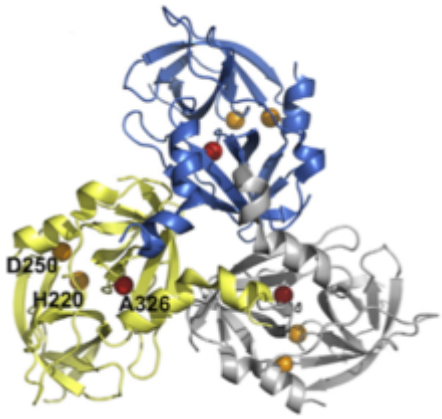

Figure 11: Crystallographic Representation of HTRA1 Trimer
HTRA1 monomers (yellow, blue, grey) can assemble into a homotrimer. The three amino acids that make up the catalytic triad are represented as orange (Aspartate 250), yellow (Histidine 220), or red (Serine 326/Alanine 326) circles [224].

1.7.2. Function

HtrA proteases were first demonstrated as playing a role in protein quality control in bacteria. In this case, they have the ability to degrade misfolded proteins with hydrophobic residues resulting from impaired processing or cell stress. HtrAs also qualify as chaperones, contributing to protein folding and inhibiting protein clustering [224]. However, these functions have not been demonstrated in mammalian cells yet, where other intracellular and extracellular tasks have been conferred to HtrA proteins [246]. In mammals, HTRA1 is involved in the stress response. Although its precise role is still not completely understood, HTRA1 secretion increases after heat shock in order to degrade heat-affected proteins [238]. *HTRA1* gene expression has also been identified in tissue and organ

development, which strengthens its importance during ECM reorganization [225,247]. For example, the human placenta exhibits a significant upregulation of *HTRA1* during endometrial preparation and first-trimester of pregnancy [248]. Additionally, it was found in mice that suppression of *Htra1* gene expression impairs placenta and tissue development [249]. Additionally, the placenta displays a high proliferation rate and stem cell activity, suggesting that HTRA1 is reacquired at the site of cellular remodelling.

1.7.2.1. Intracellular

The majority of the HTRA1 pool is secreted due to its SP domain, but a small fraction is found within the cell. Recently, it has been suggested that the intracellular fraction of HTRA1 is secreted first and is taken back up by the cell through mechanisms appearing to be specific to HTRA1 [250]. Within the mammalian cell, several targets of HTRA1 have been identified, but HTRA1's precise function is still not clear. It was found that cell migration was inhibited when *HTRA1* was overexpressed, which indicates that HTRA1 possibly acts as a tumour-suppressor [251]. Additionally, it was suggested that low concentrations of HTRA1 could stabilize microtubule polymerization and that HTRA1 degrades tubulin *in vitro* [252].

Furthermore, tuberous sclerosis complex 2 (TSC2) has been identified as a substrate for HTRA1. TSC2 is a key player in embryogenesis, in which it regulates tissue development. HTRA1-medidated degradation of TSC2 inhibited its downstream effectors [253].

Researchers have suggested that HTRA1 is involved in apoptosis. They observed that the impairment of cell adherence activates HTRA1 proteolytic activity, leading to the degradation of inhibitors of apoptosis proteins (IAPs) [254]. Other than apoptosis, HTRA1 has also been shown to regulate anoikis, another type of cell death induced through activation of the epidermal growth factor receptor (EGFR)/Akt pathway [255].

Finally, HTRA1 exhibits the ability to cleave pro-TGF-β1, which leads to the latter's degradation [256]; however, HTRA1's interaction with TGF-β signalling elements *in vitro* remains controversial.

1.7.2.2. Extracellular

The majority of the HTRA1 pool is secreted, suggesting that HTRA1 has many more targets outside of the cell.
It is known that HTRA1 degrades numerous components of the ECM [257]. Therefore, it is reasonable to assume that HTRA1 must be tightly regulated during tissue development and regeneration to maintain tissue homeostasis. The number of HTRA1 substrates is growing steadily, and includes aggrecan [258], type II collagen [259], fibronectin [260], bone sialoprotein (IBSP), matrix Gla protein [92], decorin [92], elastin, biglycan, syndecan-4 and glypican-4 [261]. Interaction with these substrates indicates that HTRA1 may have a central role in mechanisms regulating cell differentiation and tissue remodelling.
Furthermore, it has been proposed that HTRA1 regulates TGF-β pathway signalling [233]. HTRA1 has been shown to cleave TGF-β receptors and inhibit their intracellular signalling [262]. However, more recent studies have suggested that *in vivo,* HTRA1 acts to promote TGF-β signalling by degrading latent TGF-β binding protein 1 (LTBP-1), thus increasing TGF-β1 accessibility [263].

1.7.3. Role in Diseases

HTRA1 participates in various biological processes and interacts with numerous components pertaining to a broad range of signalling pathways. For these reasons, HTRA1 has been linked to several human diseases.

1.7.3.1. AMD

Age-related macular degeneration (AMD) involves damage of the macula of the retina and can lead to blindness. The underlying reason is still unclear, but some features involving HTRA1 have already been proposed [264,265]. First, the loss-of-function of HTRA1 resulting from genetic mutations inhibit the interaction between HTRA1 and TGF-β signalling pathways and lead to retina degeneration [266]. A model in which HTRA1 expression is up-regulated due to a mutation in its promoter has also been proposed. As a consequence, the anarchic degradation of the retina's ECM by HTRA1's increased protease activity may

trigger structural changes in the ECM and contribute to the progression of AMD [267].

1.7.3.2. Cancer

Involvement of ECM rearrangement is considered to have a major impact on cancer. Because of its ability to degrade ECM components and regulate cell death, HTRA1 has been linked to oncogenesis. In particular, HTRA1 is thought to act as a tumour suppressor based on its expression being down-regulated in several cancer cell lines [239,268]. This concept is reinforced by HTRA1's involvement in numerous intracellular functions and its ability to interact with the cytoskeleton as previously described. Indeed, a decrease in HTRA1 has been linked with high resistance to chemotherapy [269] due to the promotion of cell migration (by means of an alteration in microtubule stability) and metastasis [270].

1.7.3.3. CARASIL

HTRA1 has also been found to play a role in neurological disorders. The cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) [271,272] is one relevant disorder. Patients with CARASIL are subject to reoccurring strokes of the brain tissue due to damaged arterioles. The molecular mechanism involving HTRA1 in CARASIL disorder is based on mutations occurring in *HTRA1* gene, which impair TGF-β signalling leading to the impairment of small blood vessels [273]. Despite the fact that CARASIL was described in only a limited number of patients, it strongly suggests that HTRA1's dysfunction can have a wide range of effects depending on the tissue type [274].

1.7.3.4. Alzheimer's Disease

Neurodegenerative diseases may also be a field of investigation for HTRA1 mechanisms of action. Grau *et al.* (2005) confirmed that HTRA1 was found in amyloid complexes in Alzheimer's patients [275]. Additionally, is was also proved that HTRA1 degrades Tau protein, the amyloid precursor responsible for the

accumulation of plaques in Alzheimer's [231]. However, these findings have raised a debate about HTRA1's involvement in such pathologies, since other studies reported no connection between Alzheimer's disease and *HTRA1* gene mutations [276].

1.7.3.5. Musculoskeletal Diseases

Musculoskeletal diseases (MSDs) affect components of the musculoskeletal system such as joints, bones, cartilage, tendons, muscles and ligaments. Currently, MSDs represent a public health concern due to the considerable number of people affected and the impact of the diseases on patients' mobility. HTRA1's ability to interact with ECM proteins potentially makes it a key factor in MSDs.

Duchenne muscular dystrophy

Muscle degeneration caused by Duchenne muscular dystrophy (DMD), which can lead to premature death, was the first MSD in which high levels of *HTRA1* gene expression were identified. It is important to highlight that muscle development and maintenance are based on the IGFBP5 activity needed for appropriate IGF-1 functions. However, a lack of IGFBP5 is a characteristic feature of DMD, and increases in HTRA1 are thought to be responsible for IGFBP5 degradation and abrogation of the downstream signalling pathway [232,277].

Intervertebral disk degeneration

Intervertebral disk (IVDs) degeneration is an age-related disorder involving the compression of the nerve root due to the narrowing of the vertebral space [278]. The severe refinement of the disk height and the increase in disk bulging are believed to be the result of deregulation in matrix remodelling [279]. Initially, HTRA1's involvement in IVD degeneration comes from the identification of a single nucleotide polymorphism, rs11200638, in the promoter of the *HTRA1* gene [280]. Additional studies investigating the role of HTRA1 in IVD degeneration showed that HTRA1 production was increased in the diseased tissue [260]. The suggested mechanism of action in IVD degeneration is the degradation of fibronectin by HTRA1 shown by the presence of C- and N-terminal fibronectin

fragments in the degenerated tissue [260] which are considered as key players in the mediation of subsequent MMP secretion [281,282].

Rheumatoid disorders

Patients suffering from osteoarthritis (OA) exhibit a degeneration of the articular cartilage, synovium and subchondral bone [283]. OA has been linked with the induction of an inflammatory response [284] which in turn is thought to play a major role in cartilage degradation through up regulation of MMPs [285]. It was found that HTRA1 was upregulated at both mRNA and protein levels in the articular cartilage of OA patients [286]. Despite the fact that several other studies supported a detrimental role for HTRA1 in OA [287-289] the underlying mechanism remains unknown. Additionally, mouse models of OA demonstrated that the increase of Htra1 in the diseased articular cartilage correlated with the increase in Mmp13 and discoidin domain-containing receptor 2 (Ddr2) [259,290], which are central players in cartilage degradation [291,292].

Another type of arthritis, rheumatoid arthritis (RA) is caused by the ability of the synovial fibroblasts to regulate inflammation and joint destruction via the secretion of MMPs [293,294]. HTRA1 was initially identified in RA by using a mouse model of collagen-induced arthritis [295]. High levels of *Htra1* mRNA were identified at sites of swollen joints and more specifically in hypertrophic chondrocytes localized in the degenerated cartilage. Furthermore, it was shown that HTRA1 was also upregulated in RA patients where it triggered the production of MMPs by the synovial fibroblasts and this process occurred in a proteolytic dependant manner [294,296]. Additionally, the increase in fibronectin fragments was reported in RA as well as in OA, suggesting that HTRA1 degrades fibronectin is MSDs. Finally, HTRA1 is also known to degrade articular cartilage components such as proteoglycans [297].

Bone physiology

The available literature indicates a growing interest in HTRA1's role in bone biology [92,262,295]. HTRA1 has already been shown to play a role in bone development since investigations using immunohistochemistry and *in situ* hybridization identified HTRA1 at early stages of development. It was expressed by MSCs in the pre-cartilage condensations and at late stages of bone formation in the osteocytes embedded in the bone matrix [233,295]. HTRA1 was also

identified together with HTRA3 at the early stages of development in mice [227]. Several studies have suggested that HTRA1 is a positive regulator of osteogenesis, as it promotes MSC differentiation and mineralization at the expense of adipogenesis [298-301], and is expressed by osteoid and in newly formed bone [227]. In support of these findings, it was reported that HTRA1 overproduction stimulated hBMSC matrix mineralization [298], while its repression inhibited MSCs differentiation toward osteoblasts [298,300,301]. HTRA1 has also been positively linked with bone formation and regeneration since HTRA1 was identified in developing bone [227,295], and its production was significantly increased in hypertrophic chondrocytes and osteoblasts at sites of newly formed bone in fracture callus [298]. Its expression at fracture sites correlated with the presence of HTRA3 [302]. Recently, further findings showed HTRA1 as having a positive role in bone formation where high levels of HTRA1 were reported in calvarial growth sites *in vivo*, correlating with an important osteoblast activity [303].

However, despite the increasing number of studies that have reported a role for HTRA1 in bone development, its actual mode of action remains controversial.

Previous *in vitro* studies established HTRA1 as a negative regulator of osteoblast-mediated matrix mineralization due to its ability to target the TGF-β signalling pathway [262]. This was further supported by *in vivo* studies, where bone microarchitecture was significantly improved in *Htra1*-knockout mice. Additionally, HTRA1 over-expression was found to have a detrimental role in BMP-2-induced differentiation of 2T3 osteoblasts as demonstrated by reductions in matrix mineralization following the exogenous addition of HTRA1 [93]. In support of this, *Htra1*-knockdown in BMP-2-treated 2T3 cells had a positive influence on osteogenesis since matrix mineralization was significantly enhanced in these cultures. Similarly, the addition of recombinant HTRA1 to the mouse KusaO cell line led to reductions in osteogenic gene markers, as well as alkaline phosphatase activity [304].

1.8 Summary

Stem cells have the ability to differentiate into various tissues and eventually, complete organs. In this way, they reflect the perfect targets for medical research in regenerative medicine. It is critical to gain a deeper understanding of stem cell

differentiation in order to improve biomedical and therapeutic applications. Thus far, it is common knowledge that osteogenesis is a process involving active stem cell differentiation and extensive remodelling of tissues [87]. Deficiencies in bone formation and repair exemplify current therapeutic challenges due to the lack of information relating to osteogenic regulators. This is the reason why the application of optimized therapies to treat bone-related diseases or fracture healing remains unsatisfactory. HTRA1 may represent a key factor in bone formation and regeneration since its involvement in MSC osteogenesis has already been confirmed [92,93]. However, data pertaining to its functional role *in vivo* is lacking.

2. Hypothesis and Aims of the Study

HTRA1's effects on bone development [305], *in vitro* matrix mineralization [92] and osteoblast differentiation [301] are well reported. Nevertheless, knowledge pertaining to how HTRA1-driven MSC differentiation may influence bone formation and regeneration remains unclear and controversial [262,263]. Therefore, the present study pursues two main aims in order to provide an in-depth assessment of HTRA1's involvement in bone development and repair:

Aim 1: Assess the effects of loss-of-function of HTRA1 on the osteochondral differentiation potential of the mouse MSC cell line C3H10T1/2.

Aim 2: Determine the effect of HTRA1 loss on cartilage and bone formation in a mouse osteotomy model.

The results gathered from this study will enhance our current knowledge regarding HTRA1's potential involvement in the osteogenic lineage commitment of MSCs *in vitro*, and provide preliminary insights into its influence over bone regeneration *in vivo.*

3. Results

3.1. Overview of published and submitted manuscripts

3.1.1. **Filliat G**, Mirsaidi A, Tiaden AN, Kuhn GA, Weber FE, Oka C, Richards PJ. Role of HTRA1 in bone formation and regeneration: In vitro and in vivo evaluation. PLoS One. 2017 Jul 21;12(7):e0181600.
DOI: 10.1371/journal.pone.0181600.

Contribution: Designed, performed and analysed most of the experiments.

3.1.2. Glanz S, Mirsaidi A, Lopez-Fagundo C, **Filliat G**, Tiaden AN, Richards PJ. Loss-of-function of *HtrA1* abrogates all-trans retinoic acid-induced osteogenic differentiation of mouse adipose-derived stromal cells through deficiencies in p70S6K activation. Stem Cells Dev. 2016 May 1;25(9):687-98.
DOI: 10.1089/scd.2015.0368

Contribution: Assistance in cell culture workload.

3.1.1 Role of HTRA1 in bone formation and regeneration: In vitro and in vivo evaluation.

PLOS ONE

RESEARCH ARTICLE

Role of HTRA1 in bone formation and regeneration: *In vitro* and *in vivo* evaluation

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Citation: Filliat G, Mirsaidi A, Tiaden AN, Kuhn GA, Weber FE, Oka C, et al. (2017) Role of HTRA1 in bone formation and regeneration: *In vitro* and *in vivo* evaluation. PLoS ONE 12(7): e0181600. https://doi.org/10.1371/journal.pone.0181600

Editor: Gianpaolo Papaccio, Università degli Studi della Campania "Luigi Vanvitelli", ITALY

Received: May 19, 2017

Accepted: July 3, 2017

Published: July 21, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by grants from the Swiss National Science Foundation (31003A_156313), Swiss Life, and the Foundation for Research in Science and Humanities at the University of Zurich.

Competing interests: The authors have declared that no competing interests exist.

Abstract

The role of mammalian high temperature requirement protease A1 (HTRA1) in somatic stem cell differentiation and mineralized matrix formation remains controversial, having been demonstrated to impart either anti- or pro-osteogenic effects, depending on the *in vitro* cell model used. The aim of this study was therefore to further evaluate the role of HTRA1 in regulating the differentiation potential and lineage commitment of murine mesenchymal stem cells *in vitro*, and to assess its influence on bone structure and regeneration *in vivo*. Our results demonstrated that short hairpin RNA-mediated ablation of *Htra1* in the murine mesenchymal cell line C3H10T1/2 increased the expression of several osteogenic gene markers, and significantly enhanced matrix mineralization in response to BMP-2 stimulation. These effects were concomitant with decreases in the expression of chondrogenic gene markers, and increases in adipogenic gene expression and lipid accrual. Despite the profound effects of loss-of-function of HTRA1 on this *in vitro* osteochondral model, these were not reproduced *in vivo*, where bone microarchitecture and regeneration in 16-week-old *Htra1*-knockout mice remained unaltered as compared to wild-type controls. By comparison, analysis of femurs from 52-week-old mice revealed that bone structure was better preserved in *Htra1*-knockout mice than age-matched wild-type controls. These findings therefore provide additional insights into the role played by HTRA1 in regulating mesenchymal stem cell differentiation, and offer opportunities for improving our understanding of how this multifunctional protease may act to influence bone quality.

Introduction

Mammalian high temperature requirement protease A1 (HTRA1) is one of four HtrA serine protease family members [1, 2], having recently come into prominence by virtue of its predicted involvement in the genetic disorders age-related macular degeneration (AMD) [3, 4]

and cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) [5, 6]. HTRA1, like its three paralogs, contains a trypsin-like protease domain and one PDZ domain [7]. At the amino acid level, HTRA1 shares highest identity with HTRA3 [8]. Furthermore, both HTRA1 and HTRA3 have been detected at comparable locations both in mice and humans [9, 10]. Although primarily regarded as a secreted protease, HTRA1 has been detected in several different subcellular locations [11–13], thus providing alternative routes through which it may influence biological processes. In this regard, HTRA1 has amassed an impressive collection of substrates, including both intracellular (e.g. tuberous sclerosis complex 2, tubulins, tau, and proTGF-β1) [11–14] and extracellular (e.g. bone sialoprotein, fibronectin, elastin, fibromodulin, TGF-β1) [15–19] proteins. Subsequently, interest in HTRA1's contribution to human development and disease is wide ranging, encompassing numerous research fields such as cancer [20, 21], reproduction [22, 23], neurology [17, 24], and the musculoskeletal system [25].

Findings from our previous studies and from others, have identified HTRA1 as an important factor in determining the lineage commitment of primary mesenchymal stem cells (MSCs), where it acted to promote osteogenesis at the expense of adipogenesis [15, 26–28]. In support of this, HTRA1 protein has been detected in developing bones *in vivo*, as well as in fracture callus [9, 15, 19]. However, in contrast to these findings, several studies now exist in which HTRA1 has been demonstrated to impart a negative influence over osteogenesis [29, 30]. Although the cause of these conflicting results remains unclear, it is important to note that inherent differences exist between the cell culture systems used in each of these studies, and may therefore indicate that cell specific effects of HTRA1 need to be taken into account. In further support of HTRA1's role in repressing osteogenesis, studies using *Htra1*-knockout mice demonstrated significant improvements in a small number of bone parameters at selected skeletal sites [31]. However, these findings are confounded by the apparent lack of any skeletal aberrations in HTRA1 deficient mice generated by other investigators [32]. Clearly therefore, HTRA1's regulation of bone formation remains a controversial issue and as such, requires further investigation.

In the current study, we have assessed the role of HTRA1 in regulating osteogenesis *in vitro* and *in vivo*. We determined the effects of loss-of-function of HTRA1 on the differentiation potential of C3H10T1/2 cells stimulated with BMP-2, and on bone development and regeneration in mice. We demonstrated that matrix mineralization was significantly enhanced in HTRA1 deficient C3H10T1/2 cells, in association with the increased expression of several osteogenic gene markers. In addition, adipogenesis was also enhanced in HTRA1 deficient C3H10T1/2 cells, whilst chondrogenic gene expression was downregulated. By contrast, HTRA1 deficiency had no effect on the bone microarchitecture or regeneration of femurs from 16-week-old mice, although bone structure in aged mice was significantly improved as compared to age-matched wild-type controls.

Materials and methods

Materials

Human recombinant bone morphogenetic protein-2 (hrBMP-2) was prepared as previously reported [33]. Polyclonal rabbit anti-HTRA1 and anti-HTRA3 were generously provided by Prof. Michael Ehrmann (University of Duisburg-Essen, Germany) [24, 34] and Prof. Chio Oka (NAIST, Japan) [9]. Biotinylated swine anti-rabbit IgG (E0431) was purchased from Dako (Baar, Switzerland).

Cell culture and differentiation

C3H10T1/2 cell line. The murine mesenchymal cell line C3H10T1/2 [35] was kindly provided by Dr. Ronald Biemann (University of Magdeburg, Germany). Cells were cultured in normal growth medium consisting of Dulbecco's modified eagle medium (DMEM-low glucose, with GlutaMAX; Thermo Fisher Scientific, Reinach, Switzerland), supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich, Buchs, Switzerland), and penicillin/streptomycin (50 units/ml; 50 μg/ml; Thermo Fisher Scientific). For differentiation studies, cells were seeded at a density of 7'000 cells/cm^2 and cultured in osteogenic induction medium consisting of normal growth medium supplemented with 10 mM β-glycerophosphate (Sigma-Aldrich), 50 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich) and hrBMP-2 (100 ng/ml) for up to 49 days with regular medium changes.

Lentiviral shRNA

Lentiviral shRNA constructs specific for *Htra1* were purchased from the Sigma Mission library (Sigma-Aldrich) and consisted of TRCN0000031484 (sh*Htra1*^{84}) and TRCN0000031486 (sh*Htra1*^{86}). The SHC002 non-target control shRNA construct (shControl) was kindly provided by Prof. Michael Ehrmann (University of Duisburg-Essen, Germany). All shRNA construct were cloned into the pLKO.1-puro vector. In order to generate shRNA-expressing lentiviral particles, HEK293T cells were transfected with shRNA plasmids, in combination with packaging plasmid pCD/NL-BH*DDD (Addgene plasmid #17531) [36] and envelope plasmid pLTR-G (Addgene #17532) [37] using calcium phosphate co-precipitation, and lentiviral particles collected at 24 and 48 h. C3H10T1/2 cell cultures were transduced with virus, together with 8 μg/ml polybrene (Sigma-Aldrich), and medium refreshed with normal growth medium after 24 h. Transduced cells were selected for 1 week in the presence of 2 μg/mL puromycin (Sigma Aldrich), and subsequently seeded at 7'000 cells/cm^2 in cell culture plates.

RT-qPCR

Reverse-transcription quantitative PCR (RT-qPCR) was performed using TaqMan Gene Expression Assays (Thermo Scientific) (S1 Table) as previously described [27]. Briefly, a total of 0.5 μg of RNA was reverse-transcribed using Superscript II (Thermo Scientific), and successive qPCR reactions performed using the StepOnePlus (Thermo Scientific). Values were normalized to *Rps12* mRNA levels and presented as fold change according to the $2^{-\Delta\Delta CT}$ method.

Animals

Mice with targeted mutations in *Htra1* were generated using homologous recombination as previously described [38]. Mice were housed in groups of two to five animals under specific pathogen free conditions, and were allowed to acclimatize for one week prior to surgery. Housing rooms were maintained on a light/dark cycle of 12/12 h with artificial light, and animals were fed a commercial diet and water *ad libitum*. All surgeries were performed under aseptic conditions using isoflurane anaesthesia, and post-operative pain controlled using Buprenorphine. Mice were euthanized by cervical dislocation following isoflurane-induced anaesthesia. All procedures were approved by the Veterinary Office of the Canton of Zurich, Switzerland (Project License 262/2014 and 197/2013) and were carried out in strict accordance with the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals.

Femoral osteotomy model

A femoral osteotomy model was performed in wild-type (WT) (n = 62) and *Htra1*-knockout (*Htra1*-KO) (n = 61) female mice (16 weeks-of-age) using previously established protocols [39, 40]. The mean weights of WT (22.7 g ± 1.5) and *Htra1*-KO (23.2 g ± 1.8) mice were not significantly different at the time of surgery, and animals were randomly assigned to experimental groups. Mice were injected subcutaneously with Buprenorphine (Temgesic® solution, 0.3 mg/mL; Reckitt Benckiser, Wallisellen, Switzerland) at a dose of 0.1 mg/kg 30 min prior to surgery, and subsequently placed under general anaesthesia using 2% isoflurane and 100% oxygen as a carrier at 400 ml/min. Eye cream was administered to the eyes to prevent drying out. The skin was incised over the lateral aspect of the thigh and a flexible or rigid 4 hole MouseFix plate (RISystem, Davos, Switzerland) secured to the anterolateral aspect of the femur using interlocking screws. A Gigli saw (0.22 mm) was then used to create a mid diaphyseal osteotomy gap with the assistance of a saw guide. The osteotomy site was then irrigated with sterile saline, and the incision closed using Vicryl 6–0 (Ethicon, Norderstedt, Germany) and Appose ULC 35W skin staples (Medtronic, Muenchenbuchsee, Switzerland). Betadine was applied topically to the wound as a preventative measure against possible infection. Post-operative pain was controlled using Buprenorphine (1 mg/kg via drinking water) *ad libitum* for the first 4 days, and animal health and well-being monitored and recorded using a comprehensive scoring system every 12 h for the first 3 days, and then three times per week for the remainder of the study. Anaesthetized mice were euthanized at 10, 14, 21 and 35 days after surgery (n = 8–13 mice/group/time point) by cervical dislocation, and femurs harvested for further analysis.

Micro-CT analysis of mouse femurs

Following the removal of surrounding soft tissue, mouse femurs were fixed in 4% formaldehyde in phosphate buffered saline (PBS, pH 7.4) for 24 h at 4˚C. Bones were then extensively washed in running tap water and stored in 70% ethanol until analysed. Comparisons of bone structure in intact femurs were performed between 16-week-old WT (n = 8), *Htra1*-heterozygous (*Htra1*-HET) (n = 8), and *Htra1*-KO (n = 7) mice; 52-week-old WT (n = 8) and *Htra1*-KO (n = 6) mice. Femurs were scanned on a microCT40 (Scanco Medical AG, Brüttisellen Switzerland) operated at 55 kVp and 145 μA with 200 ms integration time and 2-fold frame averaging. Images were reconstructed from 1000 projections at a nominal isotropic resolution of 10 μm. After application of a Gaussian Filter (sigma 0.8, support 1), image thresholds were set, and automated masks of full bone, cortex and metaphyseal trabecular bone created [41, 42].

Evaluation of bone volume in osteotomy sites stabilized with a flexible MouseFix plate was performed in WT and *Htra1*-KO mice at 21 days (WT, n = 11; *Htra1*-KO, n = 10) and 35 days (WT, n = 10; *Htra1*-KO, n = 13) post-surgery. Analysis of osteotomy sites stabilized with a rigid MouseFix plate was performed in WT and *Htra1*-KO mice at 21 days (WT, n = 9; *Htra1*-KO, n = 9) post-surgery. Following removal of the MouseFix plate, micro-CT measurements were performed using the same settings as the intact femurs described above. After image processing, a threshold of 25% of maximum grey value was applied, and a volume of interest (500 x 500 x 280 voxels) manually selected to accommodate the full callus volume between the inner screws in which the volume of mineralized tissue was calculated (S1 Fig). We chose not to distinguish between original and newly formed bone as no reliable thresholds could be determined. Analysis of osteotomy repair was not performed in cases where the MouseFix plates failed to attach correctly during surgery (WT, n = 6; *Htra1*-KO, n = 7), or showed signs of loosening or dislocation at the time of harvesting (WT, n = 4; *Htra1*-KO, n = 4).

Histological staining of C3H10T1/2 cell cultures

Alizarin Red S. Matrix mineralization was assessed using Alizarin Red S staining as previously described [27]. Cells were washed in phosphate buffered saline (PBS) and fixed in 4% formaldehyde in PBS (pH 7.4) for 1 h at room temperature. Cells were then washed in water and stained with 2% Alizarin Red S (pH 4.2) for 10 min at room temperature. Cells were subsequently washed in PBS, and images captured using a digital camera (Canon EF-S18-55IS2). Alizarin Red S was then extracted in 10% cetylpyridinium chloride (Sigma-Aldrich), and optical densities measured at 570 nm using an Infinite M200 multiplate reader (Tecan) and normalized to cell number as previously described [27].

Oil Red O. Lipid accrual was visualized by Oil Red O staining according to previously published protocols [26]. Briefly, cells were washed in PBS and fixed in 4% formaldehyde in PBS (pH 7.4) for 1 h at room temperature. Cells were then rinsed in 60% isopropanol and after drying, stained with 0.3% Oil Red O in isopropanol for 10 min at room temperature. Cells were subsequently washed in water and images captured using a digital camera (Canon EF-S18-55IS2).

Histological analysis of mouse bone

Mouse femurs were collected at selected time points following osteotomy and fixed in 4% paraformaldehyde in PBS (pH 7.4) for 24 h at 4˚C. Bones were subsequently washed in running tap water and incubated in decalcifying solution consisting of 15% ethylenediaminetetraacetic acid (EDTA) (pH 8) with 0.5% paraformaldehyde for up to 2-weeks at 4˚C with regular changes [39]. Once decalcified, bones were washed in running tap water overnight, processed and embedded in paraffin wax.

Safranin O/ Fast Green. Safranin O/ Fast Green staining was used to visualize cartilaginous callus formation in paraffin wax sections (8 μm) of femurs at 10, 14 and 21 days following osteotomy. Tissue sections were dewaxed, rehydrated and stained in 0.05% Fast Green for 5 min. Slides were directly transferred to 0.1% Safranin O for 5 min and following dehydration in graded alcohols, mounted in DPX. Images were captured using a Leica M205C stereo microscope (Leica Microsystems, Heerbrugg, Switzerland) fitted with a digital camera (Canon EF-S18-55IS2). The ratio of Safranin O positive area to callus area was determined using NIH ImageJ software, where at least three serial tissue sections of the central callus region between the inner screws from 7 to 10 mice per group were analysed.

Immunohistochemistry. Tissue sections were dewaxed, rehydrated and treated sequentially with Avidin/Biotin Blocking Kit (Abcam), 3% H_2O_2 and normal swine serum (Reactolab, Servion, Switzerland) to reduce non-specific staining. Sections were then incubated for 1 h at 37˚C with either polyclonal rabbit anti-HTRA1 (1:300), polyclonal rabbit anti-HTRA3 (1:400), or equivalent dilutions of normal serum. After washing in PBS, sections were incubated with a biotinylated swine anti-rabbit IgG (1:400) for 45 min at 37˚C followed by washing and a further incubation for 30 min with Vectastain (Reactolab). Sections were developed using 3,3' diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich), counterstained with Harris modified hematoxylin (Sigma-Aldrich) and visualized using an Olympus BX51 light microscope (Olympus Schweiz AG, Volketswil, Switzerland).

Statistical analysis

Two-tailed unpaired Student's *t*-test was used for comparison of two groups, and one-way analysis of variance (ANOVA) with Tukey's post hoc test was used for multiple group comparisons. In all cases, a *P*-value of < 0.05 was considered statistically significant, and all data were expressed as mean ± standard deviation (S.D.).

Results

Loss-of-function of HTRA1 enhances osteogenic differentiation of C3H10T1/2 cells

The multipotent mouse cell line C3H10T1/2 represents a useful tool for investigating osteogenesis *in vitro*, having the capability of simulating many of the characteristics associated with endochondral ossification in response to BMP-2 stimulation [43, 44]. In the current report, we used lentivirus-delivered shRNA targeting the *Htra1* gene to assess the influence of loss-of-function of HTRA1 on BMP-2-induced osteochondral differentiation of C3H10T1/2 cells over the course of 7 weeks. We observed a time dependent increase in *Htra1* expression in response to BMP-2 in C3H10T1/2 cells treated with shRNA control vector (shControl), reaching a maximum of 5.6-fold (± 0.5) at day 28 (Fig 1). Attempts were also made to measure *Htra3* and *Htra4* expression levels, but values remained below detectable limits. Transduction of C3H10T1/2 cells with *Htra1*-specific shRNA (sh*Htra1*^{84}) efficiently suppressed *Htra1* gene expression throughout the course of the study, and significantly altered the temporal gene expression profiles of selected chondrocyte and osteogenic markers in response to BMP-2 stimulation. Expression levels of the chondrogenic markers *Sox9*, *Acan*, *Col2a1* and *Col10a1* were significantly reduced in sh*Htra1*^{84}-treated cells as compared to shControl-treated cells at the majority of time points tested. By contrast, the expression levels of several osteogenic markers including *Col1a2*, *Runx2*, *Spp1*, *Sparc*, and most notably, *Bglap* and *Mmp13*, were significantly enhanced at selected time points in HTRA1 deficient cells. Interestingly, BMP-2 induced *Ibsp* expression appeared to be delayed in HTRA1 deficient cells, and was significantly lower than shControl-treated cells at day 21. However, by day 28, *Ibsp* expression levels in sh*Htra1*^{84}-treated cells had increased, and were significantly enhanced as compared to shControl cells.

It therefore appeared that loss of HTRA1 favoured a more osteogenic lineage commitment of C3H10T1/2 cells in response to BMP-2. In accordance with this, *Htra1* knockdown also significantly enhanced mineralized matrix deposition at day 42 and 49 as determined by Alizarin Red S staining (Fig 2). Similar effects were also observed when C3H10T1/2 cells were transduced with an alternative *Htra1*-specific shRNA oligonucleotide (sh*Htra1*^{86}) (S2 Fig). These data therefore confirm that HTRA1 loss acts to promote C3H10T1/2 osteogenesis and matrix mineralization.

During the course of these studies, we noticed what appeared to be adipocytes present within C3H10T1/2 cell cultures treated with sh*Htra1*^{84} following 3 to 4 weeks of stimulation with BMP-2. Indeed, Oil Red O staining confirmed the presence of numerous lipid laden cells in C3H10T1/2 cultures transduced with sh*Htra1*^{84} (Fig 3A) as compared to those transduced with shControl (Fig 3B). Furthermore, expression levels of several adipogenic markers including *Pparg*, *Fabp4*, *Cd36* and *Adipog* were significantly increased in sh*Htra1*^{84}-treated cells (Fig 3C). These results therefore demonstrated that the stimulatory effects of loss-of-function of HTRA1 on C3H10T1/2 osteogenesis were paralleled by increases in adipocyte formation.

Bone structure and regeneration are unaffected in 16-week-old HTRA1-deficient mice

Having identified HTRA1 as a mediator of osteochondral differentiation *in vitro*, we next asked the question whether these effects were also apparent *in vivo*. In order to investigate this, we used a well established *Htra1*-null mouse strain generated through targeted mutation of exon 1 [22, 38]. Unexpectedly, we failed to identify any significant differences in trabecular or cortical bone structure between the femurs of wild-type (WT), heterozygous (*Htra1*-HET) and

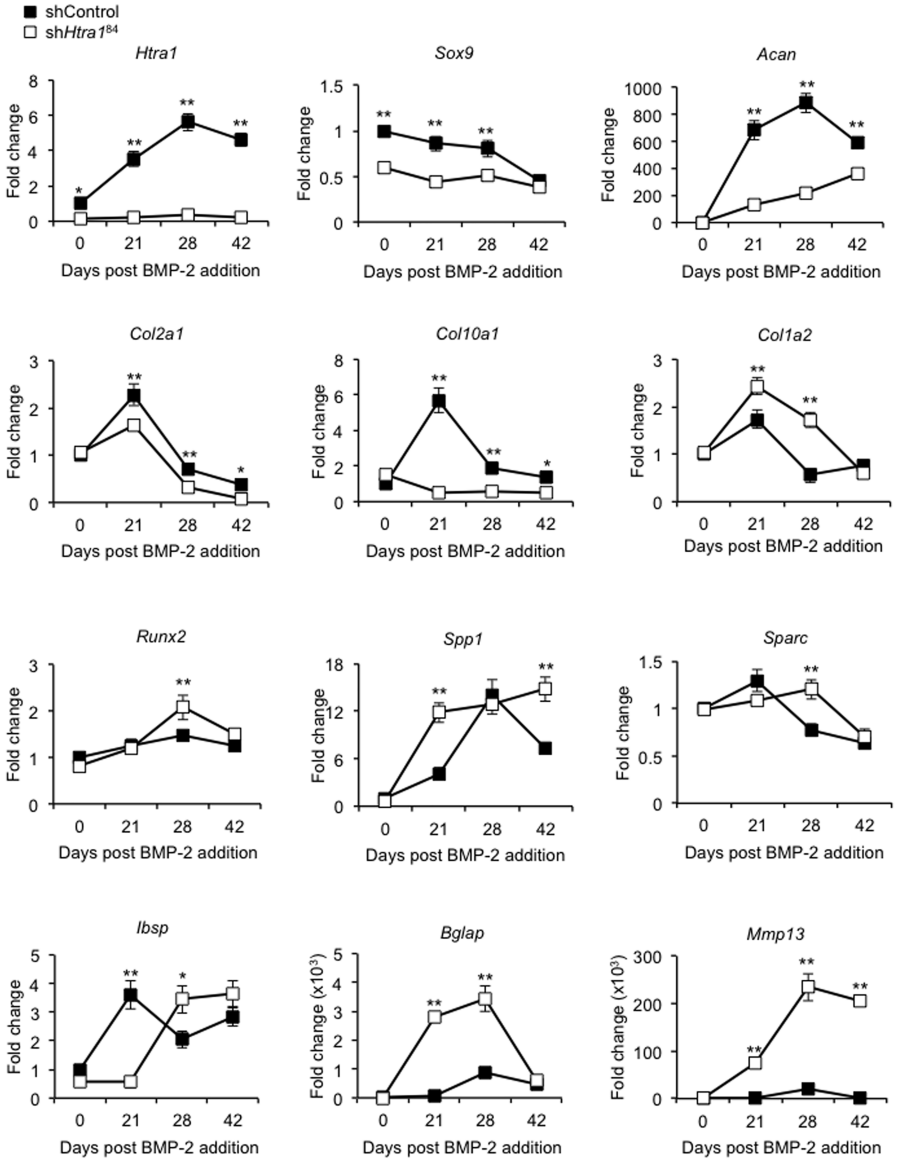

Fig 1. Effect of *Htra1* knockdown on gene expression in BMP-2 stimulated C3H10T1/2 cells. RT-qPCR analysis was used to determine the expression levels of *Htra1*, *Sox9*, *Acan*, *Col2a1*, *Col10a1*, *Col1a2*, *Runx2*, *Spp1*, *Sparc*, *Ibsp*, *Bglap* and *Mmp13* in C3H10T1/2 cells transduced with non-target control shRNA (shControl) or *Htra1*-specific shRNA (sh*Htra1*^{84}) at selected time points following stimulation with rhBMP-2 (100 ng/ml). Gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method and presented as fold change relative to uninduced cells at day 0 (value = 1). All values are expressed as mean ± S.D. (triplicates). $^{*}P < 0.05$, $^{**}P < 0.01$ comparison between shControl and sh*HtrA1*^{84} using one-way ANOVA.

https://doi.org/10.1371/journal.pone.0181600.g001

homozygous (*Htra1*-KO) *Htra1*-knockout mice, as determined by micro-CT (Fig 4). Therefore, it appeared that normal bone development, at least in mice, was not dependent on functional HTRA1.

As with long bone development, fracture healing involves a well coordinated series of events mediated, in part, through the actions of chondroprogenitor and osteoprogenitor cells, culminating in the production and eventual mineralization of a hyaline cartilage matrix [45]. However, in contrast to skeletal development, bone repair relies heavily on inflammatory cues

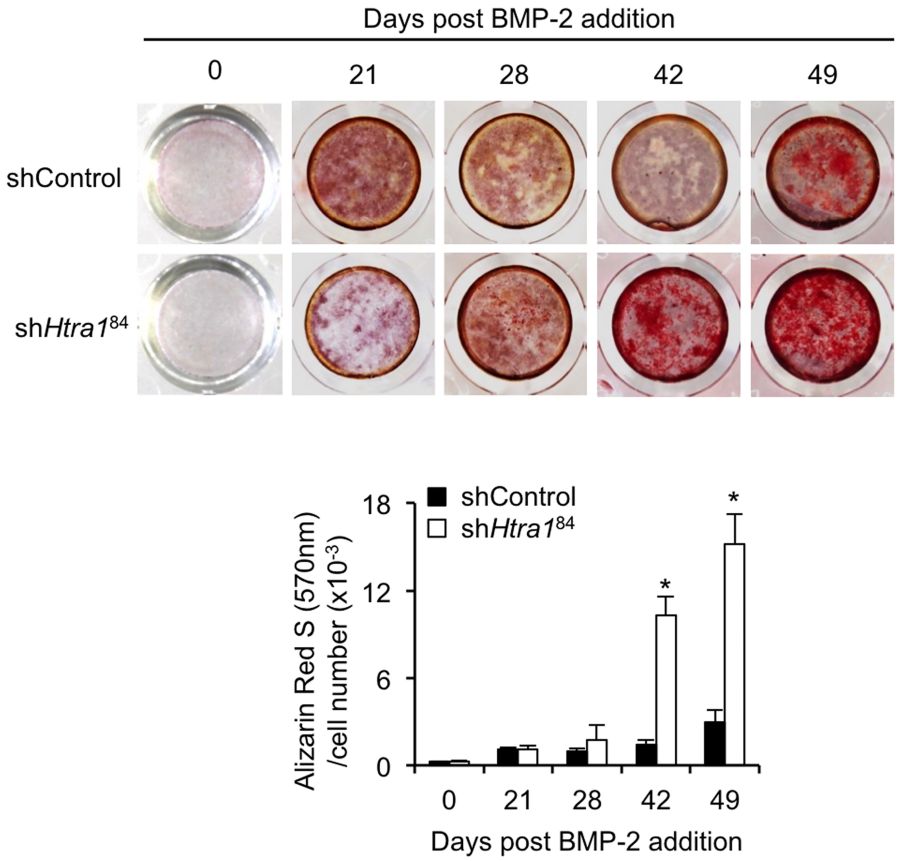

Fig 2. Effect of *Htra1* knockdown on mineralized matrix production in BMP-2 stimulated C3H10T1/2 cell cultures. CH310T1/2 cells stably transduced with non-target control shRNA (shControl) or *Htra1*-specific shRNA (sh*Htra1*^{84}) were stimulated with rhBMP-2 (100 ng/ml) for up to 49 days and stained with Alizarin Red S. The extracted dye was quantified and normalized to cell number. All values are expressed as mean ± S.D. (triplicates). *$P < 0.01$, as compared to shControl using one-way ANOVA.

https://doi.org/10.1371/journal.pone.0181600.g002

to generate the prerequisite mesenchymal stem cell condensations responsible for initiating the cartilaginous template and subsequent mineralized matrix [46]. Clearly, therefore, important differences exist between bone development and repair, which could act to influence HTRA1's impact on new bone formation. With this in mind, we next proceeded to investigate the effects of HTRA1 ablation on bone repair. An osteotomy defect was generated in the femurs of WT and *Htra1*-KO mice, and stabilized with a flexible MouseFix plate in order to promote endochondral ossification, and thus allow for the visualization of cartilage and bone formation. Surgical intervention was well tolerated by both mouse strains, with no adverse events observed, and weight loss remaining within acceptable limits (< 15% total body weight). Histological analysis of paraffin wax tissue sections using Safranin O/ Fast green demonstrated comparable amounts of cartilage callus in the osteotomy defects of WT and *Htra1*-knockout mice at days 10, 14 and 21 (Fig 5A). Similarly, analysis of osteotomy defect sites at days 21 and 35 using micro-CT identified comparable amounts of mineralized bone between both mouse strains (Fig 5B). Additional osteotomy studies were also undertaken using a rigid MouseFix plate in order to determine whether HTRA1 loss affected bone repair under conditions more conducive to intramembranous ossification. However, bone volume within rigid stabilized osteotomy sites was also found to be comparable between both mouse strains,

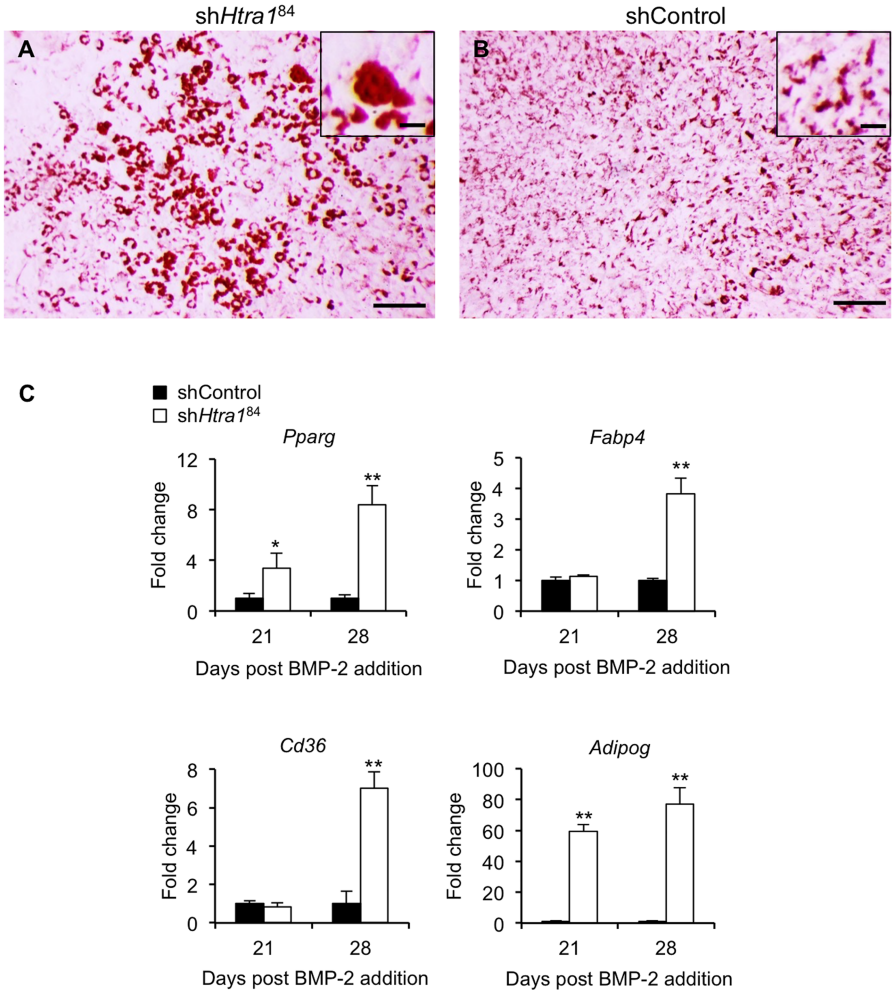

Fig 3. *Htra1* knockdown enhances C3H10T1/2 adipogenesis. CH310T1/2 cells stably transduced with *Htra1*-specific shRNA (sh*Htra1*^{84}) (A) or non-target control shRNA (shControl) (B) were stimulated with rhBMP-2 (100 ng/ml) for 23 days and stained with Oil Red O. Scale bar = 500 μm; inset scale bar = 25 μm. (C) RT-qPCR was used to measure expression levels of *Pparg*, *Fabp4*, *Cd36* and *Adipog* after 21 and 28 days stimulation with rhBMP-2 (100 ng/ml). All values are expressed as mean ± S.D. (triplicates). $*P < 0.05$, $**P < 0.01$ comparison between shControl and sh*HtrA1*^{84} using Student's *t*-test.

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indicating that the lack of deviations in bone regeneration in *Htra1*-KO mice was not due to the method of osteotomy stabilization (S3 Fig).

Immunohistological analysis of tissue sections of the osteotomy site in *Htra1*-KO mice revealed positive staining for HTRA1 protein predominantly in cells surrounding the callus cartilage at day 14 (Fig 6A), as well as in chondro-osseous transition zones at day 21 (S4A Fig). As anticipated, HTRA1 protein was not detected in any of the tissue sections analysed from *Htra1*-KO mice (Fig 6B and S4B Fig). Based on the close structural, and potentially functional similarities between HTRA1 and HTRA3 [8], we also assessed the expression of HTRA3 in osteotomy sites. Indeed, we could detect HTRA3 protein at day 14 and day 21 in sections from WT (Fig 6C and S4C Fig) and *Htra1*-KO mice (Fig 6D and S4D Fig). Furthermore, HTRA1 and HTRA3 were detected at comparable locations in the osteotomy sites of WT mice, and in some cases were even expressed by the same cell populations, thereby suggesting possible overlapping functions (inset Fig 6A and 6C; inset S4A and S4C Fig).

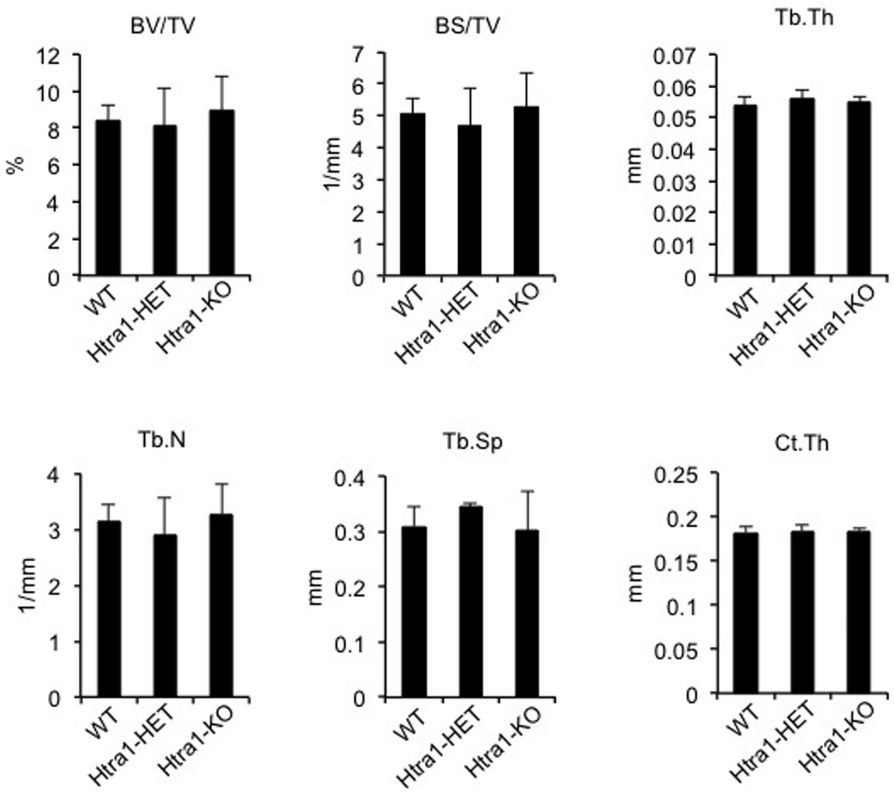

Fig 4. Mouse femur microstructure is unaffected by HTRA1 deficiency. Micro-CT analysis of femurs from 16-week-old wild-type (WT, n = 8), *Htra1*-heterozygous (*Htra1*-HET, n = 8), and *Htra1*-knockout (*Htra1*-KO, n = 7) mice. BV/TV, trabecular bone volume fraction; BS/TV, trabecular bone surface density; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular spacing; Ct.Th, cortical thickness. All results are expressed as mean ± S.D.

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HTRA1 influences the aging bone phenotype

It has previously been demonstrated that mouse embryonic fibroblasts harvested from *Htra1*-KO mice are more resistant to premature cell senescence than wild-types [47], thereby providing a possible link between HTRA1 activity and age-related processes. This is further supported by the recent finding that significant increases in systemic levels of HTRA1 were associated with increased incidences of frailty in elderly patients [48]. We therefore asked the question whether aging had any influence on the bone phenotype of *Htra1*-KO mice. Indeed, micro-CT analysis demonstrated significant improvements in trabecular and cortical parameters of femurs from 52-week-old *Htra1*-KO mice as compared to age-matched wild-type mice (Fig 7 and S2 Table and S5 Fig). Although significant increases in trabecular thickness were observed in wild-type mice, trabecular spacing was found to be significantly lower in *Htra1*-KO mice, thereby indicating that the improved trabecular spacing in these mice was primarily due to increases in trabecular number.

Discussion

Despite several studies having identified HTRA1 in calcified tissue, its actual role in bone formation continues to remain an enigma. In the current report, we set out to evaluate the effects of long-term HTRA1 depletion on bone formation both *in vitro* and *in vivo*. Our findings

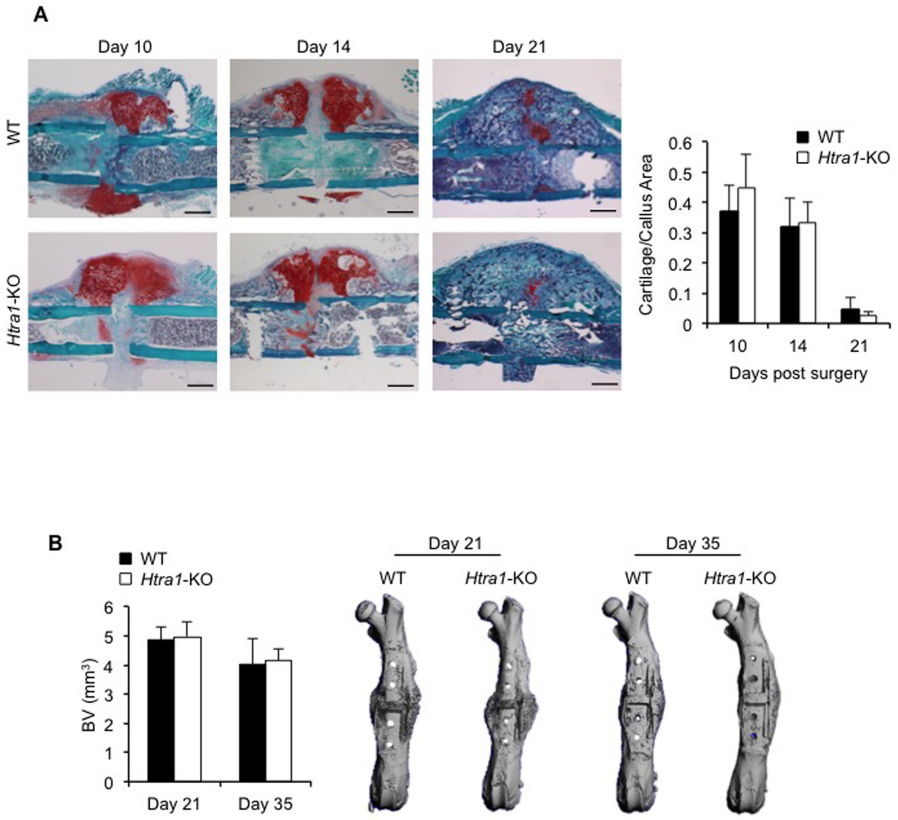

Fig 5. Bone repair in mice is unaffected by HTRA1 deficiency. (A) Cartilage area within osteotomy calluses of wild-type (WT) and *Htra1*-knockout (*Htra1*-KO) mice was determined by Safranin O staining (*red*) at 10 days (WT, n = 8; *Htra1*-KO, n = 9), 14 days (WT, n = 10; *Htra1*-KO, n = 7), and 21 days (WT, n = 9; *Htra1*-KO, n = 7) after surgery. (B) Micro-CT evaluation of bone volume (BV) in osteotomy sites of femurs stabilized with a flexible MouseFix plate from wild-type (WT) and *Htra1*-knockout (*Htra1*-KO) mice at 21 days (WT, n = 11; *Htra1*-KO, n = 10) and 35 days (WT, n = 10; *Htra1*-KO, n = 13) after femoral osteotomy. All results are expressed as mean ± S.D.

https://doi.org/10.1371/journal.pone.0181600.g005

demonstrated that although HTRA1 deficiency had a profound effect on osteogenic lineage commitment *in vitro*, it failed to influence bone structure and regeneration when assessed in 16-week-old mice. Interestingly however, significant improvements in various structural bone parameters were observed in 52-week-old HTRA1-deficient mice as compared to age-matched wild-type controls, thereby providing a possible link between HTRA1 expression and the bone aging phenotype.

Indications that HTRA proteins may play a role in regulating bone formation initially came from developmental studies performed in mice, where *in situ* hybridization and immunohistochemical analyses identified HTRA1 and HTRA3 within bone tissue [9, 19, 49]. However, it wasn't until several years later that evidence emerged of a possible functional role for HTRA1 in bone formation. Studies performed using mouse-derived osteoblasts demonstrated that despite its upregulation in response to BMP-2, HTRA1 acted as a negative regulator of bone formation [29, 30]. Moreover, the observation that *Htra1*-deficient mice have a moderately improved bone phenotype [31], suggested that HTRA1's influence on bone may go beyond simply affecting mineral formation *in vitro*. However, an equal number of studies also now exist in which HTRA1 has been shown to have a positive influence on matrix mineralization

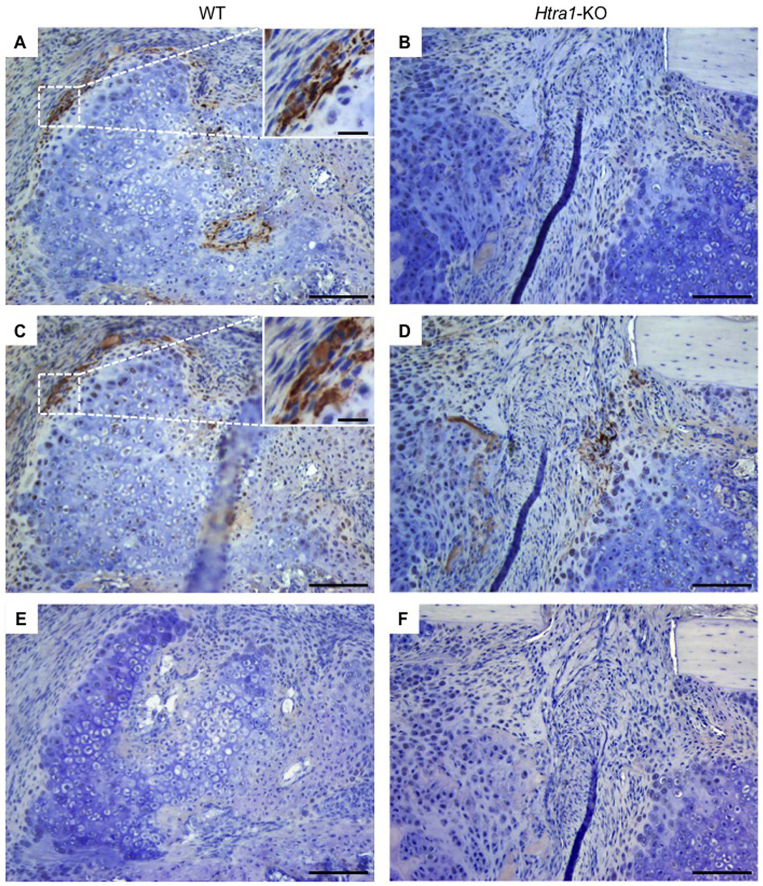

Fig 6. Immunostaining of HTRA1 and HTRA3 in callus tissue. Representative micrographs of anti-HTRA1 (A, B), anti-HTRA3 (C, D), or normal rabbit serum (E, F) stained paraffin wax sections of femurs from WT (A, C, E) and *Htra1*-KO (B, D, F) mice 14 days after osteotomy. HTRA1 and HTRA3 staining was detected using horseradish peroxidase-diaminobenzidine (*brown*) and sections counterstained with Harris modified hematoxylin (*blue*). Main scale bar = 100 μm; inset scale bar = 20 μm.

https://doi.org/10.1371/journal.pone.0181600.g006

in vitro. We and others have previously reported that loss-of-function of HTRA1 in primary human and mouse MSCs results in impaired osteogenic differentiation [15, 27, 28]. Furthermore, the overexpression of HTRA1, or its exogenous addition, had the capacity to significantly enhance matrix mineralization by MSC-derived osteoblasts [15, 28]. A major difference between these studies and those in which HTRA1 was reported to negatively influence osteogenesis, is that primary MSCs were used as opposed to immortalized cell lines (2T3 cells) or long-term bone marrow cultures (KusaO cells). As such, HTRA1's potential to modify osteogenic differentiation and bone mineral formation may be dependent on cell type.

In the current report, we have extended these investigations to include the murine mesenchymal cell line C3H10T1/2. In contrast to our previous findings, we demonstrated that loss-of-function of HTRA1 promoted C3H10T1/2 osteogenic differentiation and matrix mineralization. One possible explanation for these conflicting results is provided by the observation that HTRA1-deficient C3H10T1/2 cells had a significantly greater tendency to undergo adipogenesis. In stark contrast to primary MSCs, including those derived from fat [50] and bone [51], the osteogenic induction of C3H10T1/2 cells is positively regulated by pro-adipogenic

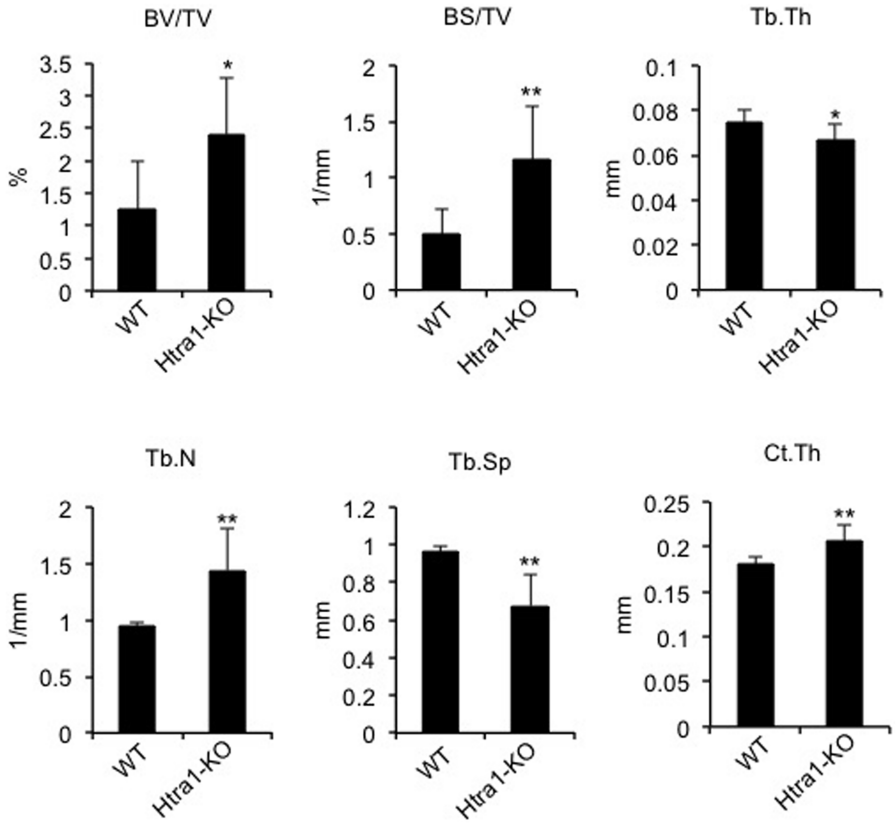

Fig 7. Femur microstructure is improved in HTRA1-deficienct mice. Micro-CT analysis of femurs from 52-week-old wild-type (WT, n = 8) and *Htra1*-knockout (*Htra1*-KO, n = 6) mice. BV/TV, trabecular bone volume fraction; BS/TV, trabecular bone surface density; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular spacing; Ct.Th, cortical thickness. All results are expressed as mean ± S.D. $*P < 0.05$, $**P < 0.01$ using Student's *t*-test.

https://doi.org/10.1371/journal.pone.0181600.g007

gene expression [52]. Therefore, it's quite possible that the stimulatory effects of HTRA1 deficiency on C3H10T1/2 osteogenesis were indirectly due to increases in the expression of adipogenic gene markers. The concept of loss-of-function of HTRA1 favouring adipogenesis has previously been demonstrated in human BMSCs [15, 26], although unlike HTRA1-deficient C3H10T1/2 cells, this resulted in significant reductions in their osteogenic potential [15]. Currently, it's unclear whether HTRA1-deficient C3H10T1/2 cells represent a heterogeneous population of adipocytes and osteoblasts, or if they are one in the same. Certainly, evidence does exist to suggest that MSC-derived adipocytes have the potential to undergo transdifferentiation into osteoblasts, and even chondrocytes [53]. Taken together, these results further exemplify the complexities involved in trying to decipher HTRA1's role in bone formation, and provide additional support for the concept that its effects on osteogenic differentiation are predominantly cell-type specific.

In order to better understand the implications of HTRA1 loss on the physiology of bone formation, several *Htra1*-knockout mouse models have been generated by different research groups. However, as with the findings from studies evaluating the effects of HTRA1 loss *in vitro*, results emanating from these *in vivo* investigations also appear to be beset by inconsistencies. Graham *et al* (2013) observed improvements in various bone structural parameters in

Htra1-knockout mice as determined by micro-CT, and concluded that this was most likely due to enhanced TGF-β signaling based on the fact that HTRA1 could cleave TGF-β receptors [31]. However, it should be pointed out that in a more recent study by Beaufort *et al* [54], HTRA1 was shown to positively regulate TGF-β pathway activation *in vivo*, although its influence on bone structure was not reported. Results from our own micro-CT analysis of intact femurs taken from 16-week-old *Htra1*-knockout mice demonstrated their bone structure to be comparable to that of age-matched wild-type mice. Moreover, despite confirming the presence of HTRA1 protein within regenerating bone of wild-type mice, and its absence from *Htra1*-knockout mouse tissue, bone repair also appeared to be unaffected by the loss of HTRA1. HTRA1's localization to, and potential involvement in, new bone formation has previously been attested to in a recent *in vivo* study examining the effects of thyroxine exposure on calvarial growth sites in mice, where enhanced levels of HTRA1 were identified at sites of increased osteoblast activity [55]. We were therefore surprised not to have observed any significant deviations in bone regeneration in HTRA1-deficient mice. Interestingly, immunohistochemical staining of regenerating bone also detected HTRA3 at similar locations as HTRA1 in wild-type mice, as well as in the callus of *Htra1*-knockout mice. As far as we are aware, this is the first report of HTRA3 within bone tissue of adult mice undergoing bone repair. As with HTRA1, it was primarily detected at the borders of cartilaginous tissue within the callus, where chondrocyte apoptosis and subsequent bone remodelling are thought to occur [56]. These findings therefore signify possible functional redundancy between these two HtrA paralogs, whereby loss of HTRA1 is compensated for by HTRA3. Further investigations into bone regeneration using mice deficient in HTRA3, or HTRA1 and HTRA3, may help to provide additional insights into the role of HtrA proteases in bone formation.

Previous studies have identified HTRA1 as an inducer of premature cell senescence [47], and elevated levels of HTRA1 have been positively correlated with increased incidences of frailty in the aged [48]. Therefore, we also considered the possibility that changes in the bone phenotype of *Htra1*-knockout mice may become more apparent with aging. Indeed, the bone structure of femurs from 52-week-old mice was significantly improved in *Htra1*-knockout mice as compared to their wild-type counterparts. These findings therefore suggest that, in mice at least, HTRA1 may represent an important determining factor for bone quality in response to aging, and further studies examining bone regeneration in aged HTRA1-deficient mice may be warranted. Certainly, these new findings are more in keeping with our *in vitro* data, where mineralized matrix formation was enhanced in HTRA1-deficient C3H10T1/2 cells. However, there still exists the matter of reconciling these observations with the results obtained from previous studies investigating the effects of loss or gain of HTRA1 function on MSC lineage commitment [15, 26–28]. The choice of cell type, and preference for immortalized cell line over primary cells, may have played some part in defining HTRA1 as a pro- or anti-osteogenic mediator. Certainly, the response of cultured cells to loss of HTRA1 varies considerably, where for instance proliferation is either decreased [57], enhanced [58], or unaffected [59] depending on the cell source used. Therefore, some caution should be taken in translating these *in vitro* findings to an *in vivo* system, where the generation of a particular phenotype may culminate from a series of heterogeneous cellular responses to alterations in HTRA1 production. Taken together, our findings further identify HTRA1 as a potent regulator of the multilineage differentiation potential of MSCs, and provide evidence to suggest that although HTRA1 does not appear to influence bone development and regeneration beyond the *in vitro* system, it may contribute to the aging bone phenotype in mice. Whether this also applies to aged human bone, however, remains to be determined.

Supporting information

S1 ARRIVE Checklist. NC3Rs ARRIVE guidelines checklist.
(PDF)

S1 Table. List of TaqMan gene expression assays used in RT-qPCR analysis.
(DOCX)

S2 Table. Full list of bone morphometric indices used in micro-CT analysis of femurs from 52-week-old mice.
(DOCX)

S1 Fig. Micro-CT analyses of bone within osteotomy site of mouse femur. Representative images of posterior, anterior, lateral and medial aspects of an *Htra1*-KO mouse femur at 21 days following osteotomy. The red colouration highlights the mineralized tissue within the volume of interest (500 x 500 x 280 voxels) as observed following removal of the MouseFix plate.
(TIF)

S2 Fig. Effect of *Htra1* knockdown on mineralized matrix production in BMP-2 stimulated C3H10T1/2 cell cultures. (A) RT-qPCR analysis was used to confirm efficient knockdown of *Htra1* gene expression in C3H10T1/2 cells transduced with *Htra1*-specific shRNA (sh*Htra1*^{86}) at selected time points following stimulation with rhBMP-2 (100 ng/ml). Gene expression levels were determined using the $2^{-\Delta\Delta CT}$ method and presented as fold change relative to uninduced cells at day 0 (value = 1). All values are expressed as mean ± S.D. (triplicates). $^{*}P < 0.01$ comparison between shControl and sh*Htra1*^{86} using one-way ANOVA. (B) CH310T1/2 cells stably transduced with non-target control shRNA (shControl) or *Htra1*-specific shRNA (sh*Htra1*^{86}) were stimulated with rhBMP-2 (100 ng/ml) for up to 42 days and stained with Alizarin Red S.
(TIF)

S3 Fig. Bone repair in mice using a rigid MouseFix plate. Micro-CT evaluation of bone volume (BV) in osteotomy sites of femurs stabilized with a rigid MouseFix plate from wild-type (WT) (n = 9) and *Htra1*-knockout (*Htra1*-KO) (n = 9) mice at 21 days after femoral osteotomy. All values are expressed as mean ± S.D.
(TIF)

S4 Fig. Immunostaining of HTRA1 and HTRA3 in callus tissue at day 21. Representative micrographs of anti-HTRA1 (A, B), anti-HTRA3 (C, D), or normal rabbit serum (E, F) stained paraffin wax sections of femurs from WT (A, C, E) and *Htra1*-KO (B, D, F) mice 21 days after osteotomy. HTRA1 and HTRA3 staining was detected using horseradish peroxidase-diaminobenzidine (*brown*) and sections counterstained with Harris modified hematoxylin (*blue*). Main scale bar = 50 μm; inset scale bar = 25 μm.
(TIF)

S5 Fig. Micro-CT analyses of femurs from 52-week-old WT and *Htra1*-KO mice. Selected images of distal femurs from wild-type (WT) and *Htra1*-knockout (*Htra1*-KO) mice illustrating the regions from which cortical (orange) and trabecular (red) bone measurements were taken. Images are representative of the median trabecular BV/TV value from each group.
(TIF)

Acknowledgments

The authors would like to express their thanks to Theresia Reding and Pawel Pelczar (University of Zurich) for their assistance with the animal studies.

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S1 Table. List of TaqMan Gene Expression Assays used in RT-qPCR analysis

Gene Symbol	Protein Product	Assay ID [a]
Htra1	High temperature requirement protease A1	Mm00479887_m1
Htra3	High temperature requirement protease A3	Mm00472631_m1
Htra4	High temperature requirement protease A4	Mm01210984_m1
Sox9	SRY-homeobox-like gene 9	Mm00448840_m1
Acan	Aggrecan	Mm00545794_m1
Col1a2	Collagen type I, alpha 2	Mm00483888_m1
Col2a1	Collagen type II, alpha 1	Mm01309565_m1
Col10a1	Collagen type X, alpha 1	Mm00487041_m1
Runx2	Runt-related transcription factor 2	Mm00501584_m1
Spp1	Secreted phosphoprotein 1	Mm01611440_mH
Sparc	Secreted protein acidic and rich in cysteine	Mm00486332_m1
Ibsp	Integrin Binding Sialoprotein	Mm00492555_m1
Bglap	Bone gamma-carboxyglutamate (gla) protein	Mm03413826_m1
Mmp13	Matrix metalloproteinase 13	Mm00439491_m1
Cd36	Cluster of differentiation 36	Mm00432398_m1
Fabp4	Fatty acid binding protein 4	Mm00445878_m1
Pparg	Peroxisome proliferator activated receptor γ	Mm00440940_m1
Adipog	Adiponectin	Mm00456425_m1
Mrps12	Mitochondrial ribosomal protein S12	Mm00488728_m1

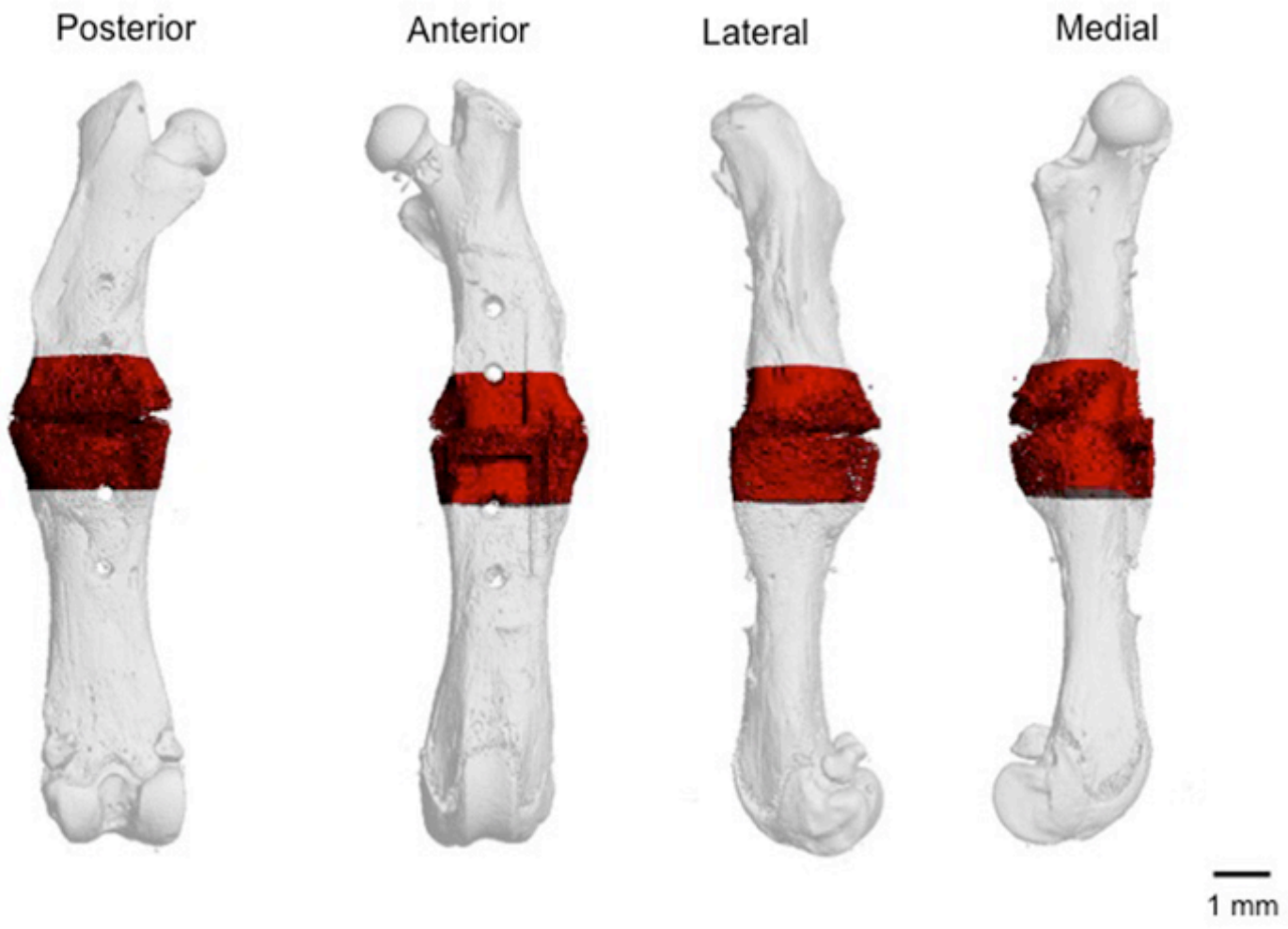
[a]TaqMan Expression Assay identity code according to supplier (Thermo Fisher Scientific, Reinach, Switzerland).

S2 Table. Full list of bone morphometric indices used in micro-CT analysis of femurs from 52-week-old mice.

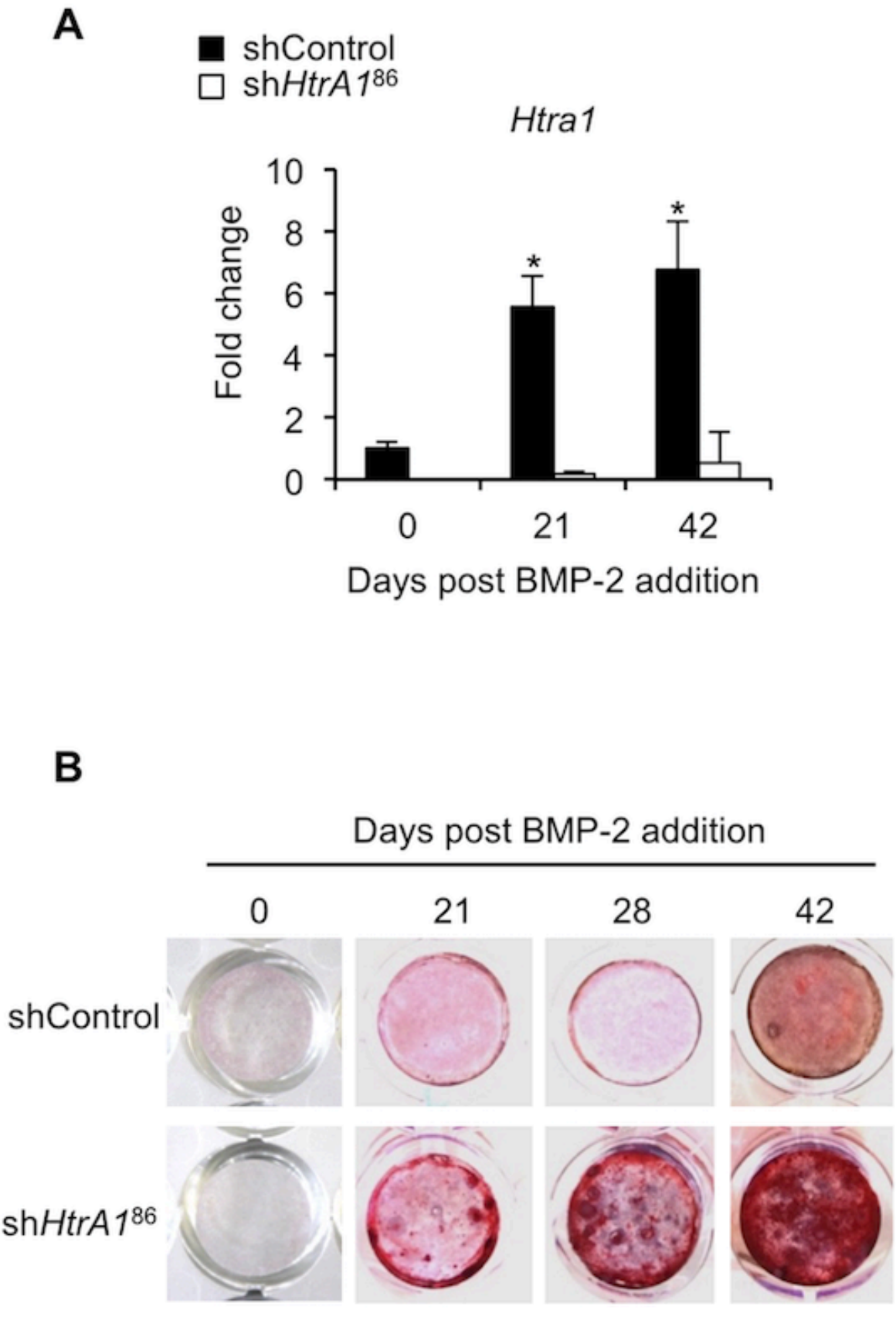
Index	WT (N=8)	*Htra1*-KO (N=6)	*P*-value[a]
AVD (%)	45.36 (± 0.34)	47.78 (± 2.38)	0.018
BV/TV (%)	1.25 (± 0.74)	2.4 (± 0.85)	0.019
BS/TV (1/mm)	0.49 (± 0.23)	1.16 (± 0.49)	0.005
BS/BV (1/mm)	41.16 (± 3.69)	47.46 (± 6.91)	0.036
Tb.Th (mm)	0.0745 (± 0.005)	0.0665 (± 0.007)	0.034
Tb.Sp (mm)	0.96 (± 0.03)	0.675 (± 0.17)	0.0004
Tb.N (1/mm)	0.95 (± 0.03)	1.44 (± 0.37)	0.0025
Ct.Ar/T.Ar (%)	40.1 (± 1.3)	45.1 (± 3.23)	0.002
Ct.Th (mm)	0.18 (± 0.008)	0.21 (± 0.02)	0.003
J (mm^4)	0.36 (± 0.04)	0.41 (± 0.02)	0.026
Imax (mm^4)	0.23 (± 0.03)	0.26 (± 0.02)	0.023
Imin (mm^4)	0.13 (± 0.01)	0.145 (± 0.01)	0.078

AVD, apparent volume density; BV/TV, trabecular bone volume fraction; BS/TV, trabecular bone surface density; BS/BV, specific bone surface; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Tb.N, trabecular number; Ct.Ar/T.Ar, cortical area fraction; Ct.Th, cortical thickness; J, polar moment of inertia; Imax and Imin, second moment of inertia; [a] statistical significance was determined using Student's *t*-test. All results are expressed as mean ± S.D.

S1 Figure



S2 Figure


A
shControl
shHtrA1^86
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10
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4
2
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Days post BMP-2 addition
B
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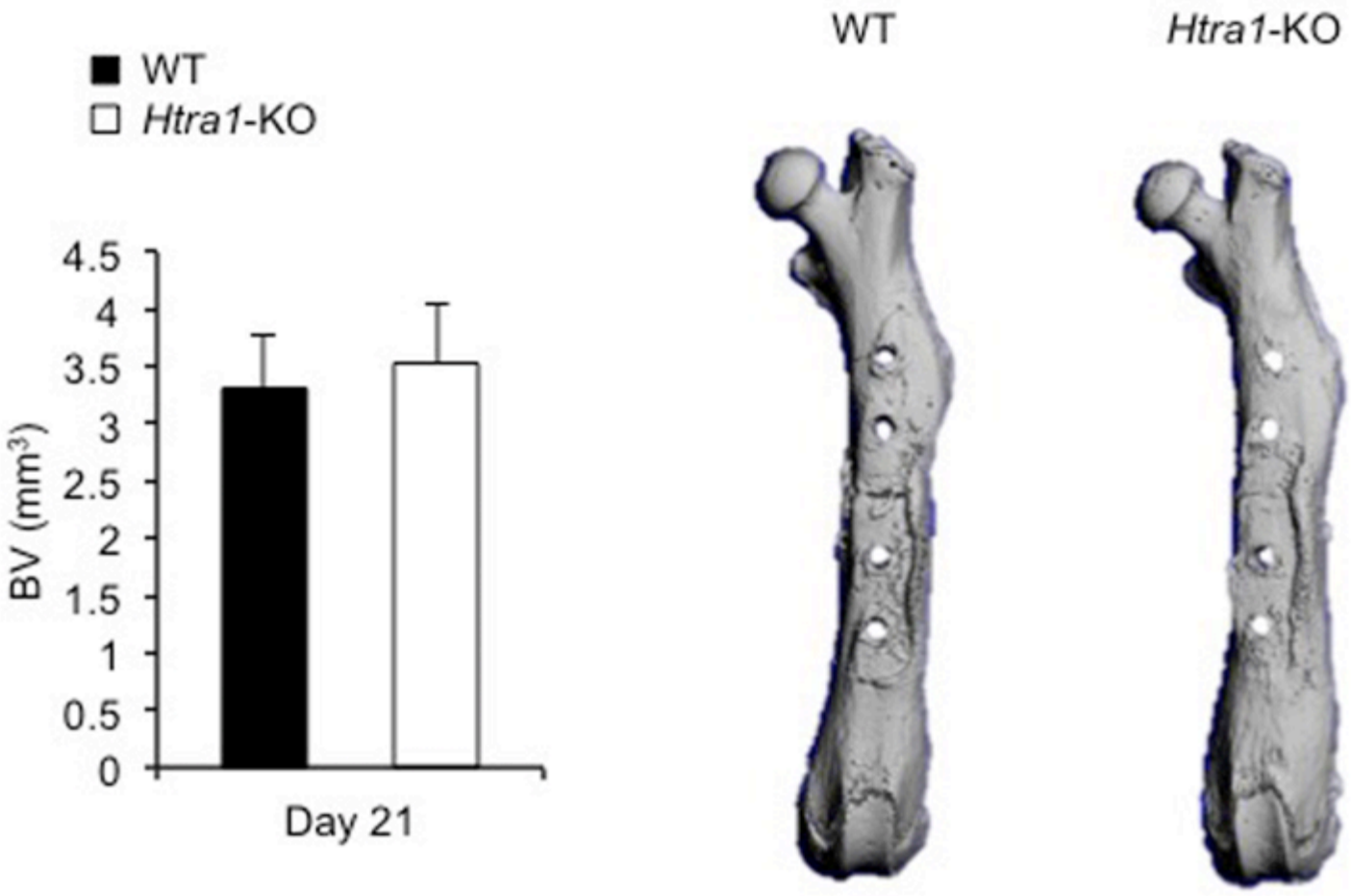
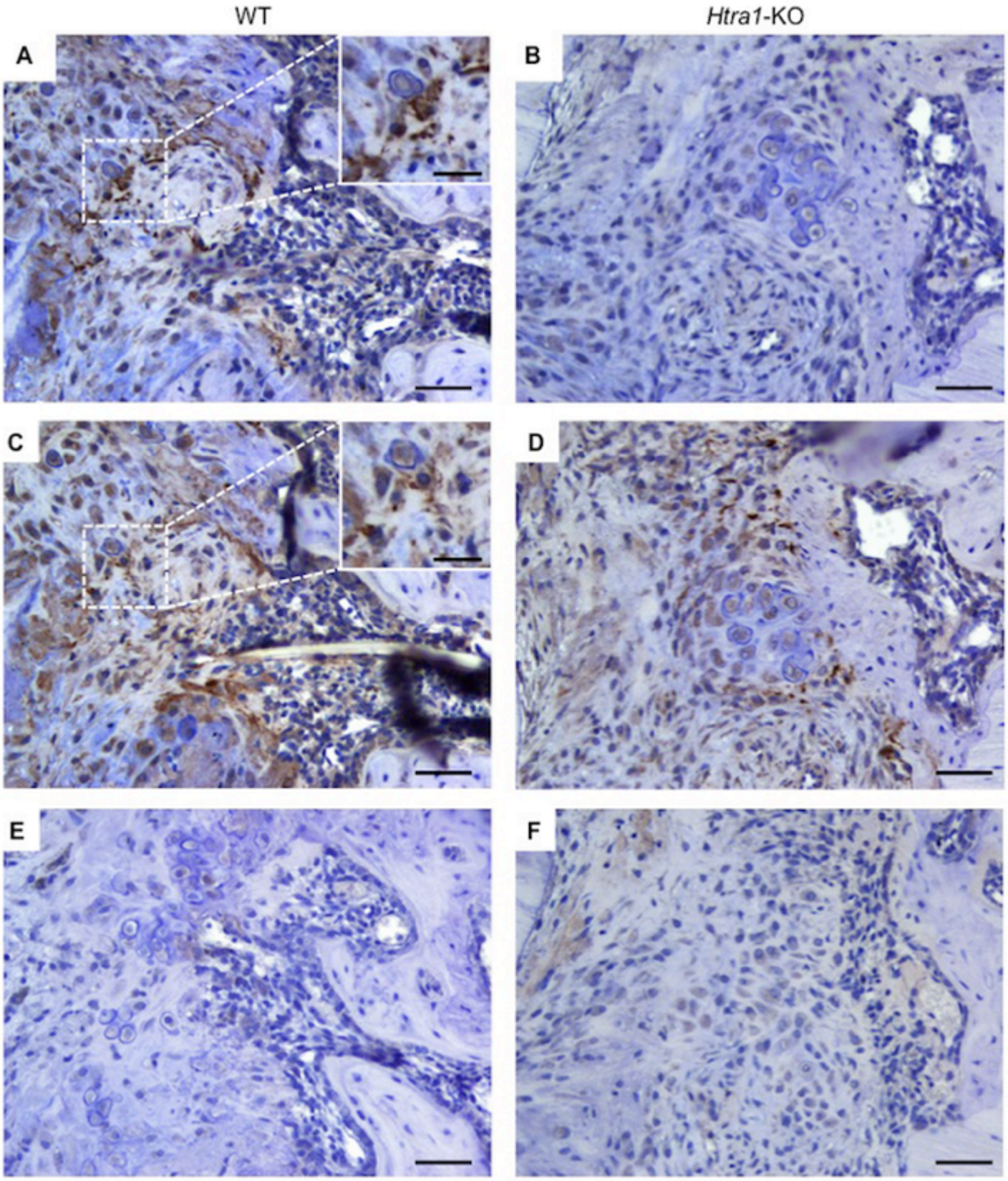
28
42
shControl
shHtrA1^86

S3 Figure



S4 Figure



S5 Figure

3.1.2 Loss-of-function of *HtrA1* abrogates all-trans retinoic acid-induced osteogenic differentiation of mouse adipose-derived stromal cells through deficiencies in p70S6K activation.

STEM CELLS AND DEVELOPMENT
Volume 25, Number 9, 2016

DOI: 10.1089/scd.2015.0368

Loss-of-Function of HtrA1 Abrogates All-*Trans* Retinoic Acid-Induced Osteogenic Differentiation of Mouse Adipose-Derived Stromal Cells Through Deficiencies in p70S6K Activation

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All-*trans* retinoic acid (ATRA) is a potent inducer of osteogenic differentiation in mouse adipose-derived stromal cells (mASCs), although the underlying mechanisms responsible for its mode of action have yet to be completely elucidated. High temperature requirement protease A1 (HtrA1) is a newly recognized modulator of human multipotent stromal cell (MSC) osteogenesis and as such, may play a role in regulating ATRA-dependent osteogenic differentiation of mASCs. In this study, we assessed the influence of small interfering RNA (siRNA)-induced repression of HtrA1 production on mASC osteogenesis and examined its effects on ATRA-mediated mammalian target of rapamycin (mTOR) signaling. Inhibition of HtrA1 production in osteogenic mASCs resulted in a significant reduction of alkaline phosphatase activity and mineralized matrix formation. Western blot analyses revealed the rapid activation of Akt (Ser473) and p70S6K (Thr389) in ATRA-treated mASCs, and that levels of phosphorylated p70S6K were noticeably reduced in HtrA1-deficient mASCs. Further studies using mTOR inhibitor rapamycin and siRNA specific for the p70S6K gene *Rps6kb1* confirmed ATRA-mediated mASC osteogenesis as being dependent on p70S6K activation. Finally, transfection of cells with a constitutively active rapamycin-resistant p70S6K mutant could restore the mineralizing capacity of HtrA1-deficient mASCs. These findings therefore lend further support for HtrA1 as a positive mediator of MSC osteogenesis and provide new insights into the molecular mode of action of ATRA in regulating mASC lineage commitment.

Introduction

Efficient osteogenic induction of mouse adipose-derived stromal cells (mASCs) is reliant on the actions of all-*trans* retinoic acid (ATRA), the carboxylic acid form of vitamin A [1–7]. This is in contrast to mouse and human bone marrow stromal cells (BMSCs), where dexamethasone is primarily used to instigate osteogenesis through upregulation of four and a half LIM domains 2 (FHL2) and activation of Wnt/β catenin signaling [8].

ATRA's ability to influence osteoblast differentiation has been observed in several different cell systems and is considered to be largely dependent on the concentration of ATRA used. While ATRA acts to enhance osteogenesis at micromolar concentrations [1–7, 9, 10], at nanomolar concentrations, it has been shown to inhibit both osteoblast gene expression and mineralization [11–13]. The concentration of ATRA used to stimulate mASC osteogenesis in vitro is generally within the range of 1–5 μM, where it acts to enhance the expression of several osteogenic markers including alkaline phosphatase (*Alpl*) and osteopontin (*Spp1*), and to induce mineralization of mASC-derived osteoblasts [3,4]. In addition, ATRA's ability to direct mASCs along the osteoblast lineage in vitro has also been exploited for the purpose of enhancing mASC-induced new bone formation in vivo. Priming of mASCs with ATRA before their implantation into mouse calvarial defects resulted in accelerated bone regeneration compared with mice treated with unstimulated mASCs [14]. However, the mechanisms through which ATRA instigates its osteogenic effects in these cells remain unclear.

Findings from studies investigating the combined effects of ATRA and bone morphogenetic protein (BMP)-2 on mASC osteogenesis suggested that ATRA's primary function was to regulate BMP signaling through enhanced BMP receptor expression [1]. However, ATRA also has the ability to induce osteogenic differentiation of mASCs in the absence of exogenous BMP-2 [2–7]. Therefore, it's likely that

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in addition to BMP signaling, ATRA targets other pathways critically involved in regulating mASC osteogenesis.

We have previously identified high temperature requirement protease A1 (HtrA1) as a novel mediator of human BMSC (hBMSC) differentiation, where it acts to enhance osteogenesis and subsequent mineralization by differentiating bone-forming cells [7]. Furthermore, *HtrA1* expression is upregulated in mASCs in response to ATRA-containing osteogenic induction medium [7].

HtrA1 is a member of the HtrA family of serine proteases and has been linked to various biological processes by virtue of its ability to interact with numerous intracellular and extracellular substrates [15]. Tuberous sclerosis complex 2 (TSC2) was the first cytoplasmic HtrA1 substrate to be identified, and its degradation by HtrA1 was shown to result in activation of the mammalian target of rapamycin (mTOR) pathway as confirmed by alterations in the phosphorylation of downstream targets eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and p70 ribosomal protein S6 kinase (p70S6K) [16]. This bears particular significance with regards to mASC osteogenesis, based on the fact that mTOR signaling plays a positive role in the osteogenic induction of several cell types including BMSCs [17–19]. However, no studies have yet sort to investigate its involvement in mediating the osteoinductive effects of ATRA on mASCs, or whether HtrA1's ability to influence mTOR signaling plays a role in determining mASC osteogenic potential.

In this study, we investigated the role of HtrA1 in the ATRA-dependent differentiation of mASCs into mineral-forming bone cells and assessed its influence over mTOR signaling events during the course of mASC osteogenesis.

Materials and Methods

Materials

Antibodies specific for nonphosphorylated Akt, mTOR, p70S6K, 4E-BP1, and rpS6; phosphorylated Akt (Ser473), mTOR (Ser2448), p70S6K (Thr389), 4E-BP1 (Thr37/46), and rpS6 (Ser235/236) were all purchased from Cell Signaling, BioConcept. Mouse monoclonal anti-tubulin was from Sigma-Aldrich. Mouse HA-probe antibody and anti-GAPDH were from Santa Cruz Biotechnology, LabForce AG. A polyclonal anti-HtrA1 antibody was generated as previously described [20]. HRP-labeled secondary antibodies specific for mouse or rabbit IgG were purchased from Jackson ImmunoResearch. Rapamycin was purchased from Enzo Life Science. BMP-2 was kindly donated by Prof. Franz Weber (University of Zurich). ATRA, dexamethasone, and PF-4708671 were from Sigma-Aldrich. The expression plasmids pRK7-HA-S6K1-F5A-E389-R3A (Addgene plasmid # 8991) and pRK7-HA-S6K1-KR (Addgene plasmid # 8985) were kind gifts from John Blenis [21].

Isolation and culture of mASCs

Primary mASCs were isolated from SAM mice as previously described [4,5]. All animal research procedures were approved by the Animal Experimentation Committee of the Veterinary Office of the Canton of Zurich, Switzerland and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals. Briefly, subcutaneous inguinal fat pads were removed and digested in Hepes buffer containing 0.1% collagenase A (Roche Diagnostics) and 0.2% bovine serum albumin for 40 min at 37°C. Adherent stromal cells were maintained in complete medium consisting of Dulbecco's modified eagle medium (DMEM-low glucose, with GlutaMAX) (Life Technologies), supplemented with 10% fetal bovine serum (FBS) (Bioswisstec) and antibiotics. Supernatant was replaced after 1 day with fresh complete medium and cells were used between passage 1 and 4 following initial analysis for mesenchymal and hematopoietic cell markers by flow cytometry as previously described [4].

Osteogenic differentiation of mASCs

mASCs were plated at 5,000 cells/cm^2 and incubated in alpha-minimum essential medium (α-MEM) (Life Technologies), supplemented with 10% FBS (Bioswisstec), 50 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 10 mM β-glycerophosphate, and either 5 μM ATRA or 100 nM dexamethasone and 100 ng/mL BMP-2 for up to 21 days with regular changes of medium as previously described [4]. Where indicated, PF-4708671 was also added to mASCs undergoing osteogenic differentiation to assess the influence of S6K1 inhibition on ATRA-dependent osteogenic induction. Alkaline phosphatase (ALP) activity was quantified in cell lysates using p-nitrophenylphosphate (pNPP) liquid substrate (Sigma-Aldrich) and values normalized to total protein content and reaction time as previously described [4]. Mineralization was visualized using Alizarin red and the amount of staining determined by measuring optical densities at 570 nm following extraction using 10% cetylpyridinium chloride (Sigma-Aldrich). Optical densities were then converted to micromoles (μM) of Alizarin red using a standard curve and normalized to cell number. The mean cell number was determined by automated counting of 4′,6-diamidino-2-phenylindole (DAPI) stained nuclei in at least six random fields of view. Images were captured on a Leica DMI 6000 inverted fluorescence microscope (Leica Microsystems). Image processing and nuclear counts were performed using NIH ImageJ software.

Quantitative reverse transcription PCR

Total RNA was isolated from mASCs and purified using TRIzol reagent (Invitrogen AG, Basel, Switzerland) according to the manufacturer's instructions. RNA (0.5 μg) was reverse transcribed to cDNA using Superscript II (Invitrogen AG) and random hexanucleotide primers (Promega AG). Quantification of mRNA expression was performed with TaqMan Gene Expression Assays (Applied Biosystems) specific for *HtrA1* (Mm00479887), *Rps6kb1* (Mm01310033), *Alpl* (Mm01187117), and *Spp1* (Mm01611440) using the StepOnePlus Real-Time PCR System (Applied Biosystems) and values normalized to *Mrps12* (Mm00488728) mRNA levels and presented as fold change according to the $2^{-\Delta\Delta CT}$ method. Each 10 μL reaction consisted of 1× TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 1× TaqMan Gene Expression Assay, and 10 ng cDNA (based upon initial RNA concentrations). All reactions were performed in triplicate in fast optical 96-well reaction plates (Applied Biosystems) at 95°C for 20 s and 40 cycles of 95°C for 1 s and 60°C for 20 s.

Western blot analysis

Total cellular protein was extracted from mASCs using CelLytic M (Sigma-Aldrich) containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich). For the analysis of HtrA1 in mASC supernatants, cells were treated for 3 days with osteogenic induction medium and then for a further 24 h in fresh FCS-free osteogenic induction medium before harvesting and concentrating supernatants 30-fold using Amicon Ultra-15, 10 kDa mwco filter units (Millipore). In each case, protein amounts were quantified using BioRad Protein Assay (BioRad). Protein samples were boiled for 5 min in loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT, and 0.002% Bromophenol blue) and equal amounts of protein loaded onto 12% SDS-PAGE gels. Protein was then electroblotted onto PVDF membranes using the Trans-Blot Turbo blotting system (BioRad) and incubated in 5% skimmed milk, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% Tween 20 (TBST) for 1 h at room temperature. Membranes were then incubated overnight at 4°C with primary antibodies specific for HtrA1 or phosphorylated or nonphosphorylated Akt, mTOR, p70S6K, rpS6, and 4E-BP1. Monoclonal mouse anti-tubulin or anti-GAPDH were used to control equal protein loading of cell lysates. Coomassie blue staining was used to control equal protein loading of cell supernatants. After washing in TBST thrice for 5 min each, membranes were incubated with a HRP-conjugated anti-mouse or anti-rabbit IgG (1:10,000) for 1 h at room temperature. Following a further washing step, peroxidase activity was detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Protein levels were quantified using NIH ImageJ software. Phosphorylated and nonphosphorylated protein values were first normalized to tubulin loading control and then the phosphorylation to total protein ratio was calculated using the normalized values.

Small interfering RNA studies

Specific knock down of gene expression was performed with Silencer Select Small interfering RNA (siRNA) (Ambion, Life Technologies) specific for *HtrA1* or *Rps6kb1* using previously described methods [7]. Briefly, mASCs (1×10^5 cells) were transfected with 20 nM of targeted siRNA or negative control siRNA (Negative Control-1) using the NEON Transfection System (Life Technologies). Following transfection, cells were seeded in cell culture plates with fresh growth medium (without antibiotics) and incubated for 24 h at 37°C, 5% CO_2. Medium was then replaced with either fresh growth medium or osteogenic differentiation medium and total RNA or protein harvested at selected time points for further analysis. The effects of siRNA mediated gene knockdown on osteogenic-induced mASC ALP activity and mineralization was determined using the ALP activity assay and Alizarin red staining respectively.

siRNA and plasmid co-transfection

mASCs were transfected with Silencer Select siRNA specific for *HtrA1* or Negative Control-1 and 1 μg of mammalian expression plasmid pRK7-HA-S6K1-F5A-E389-R3A (constitutively active p70S6K), pRK7-HA-S6K1-KR (kinase inactive p70S6K), or empty control plasmid pcDNA3 using the NEON Transfection System as described above. After 24 h, cells were induced to undergo osteogenesis and mineralization quantified after 21 days using Alizarin red staining as described above.

Statistical analysis

Two-tailed unpaired Student's *t*-test for comparison of two groups or one-way analysis of variance (ANOVA) with Tukey's post hoc test for multiple group comparisons were performed using SPSS19.0 (SPSS, Inc.). In all cases, a *P*-value of <0.05 was considered statistically significant.

Results

We have previously demonstrated that HtrA1 plays a vital role in the regulation of osteogenesis in hBMSCs [7]. In the current report, we further investigated this property of HtrA1 in ATRA-stimulated mASCs and aimed to establish its role in regulating mASC osteogenesis and mASC-derived osteoblast mineralization.

HtrA1 deficiency impairs ATRA-mediated mASC osteogenic differentiation

To examine the influence of loss-of-function of HtrA1 on the osteogenic capacity of mASCs in response to treatment with osteogenic medium containing ATRA, we analyzed mineral production by mASC-derived osteoblasts, and ALP expression and enzyme activity. Analysis of HtrA1 in mASC supernatants by western blot confirmed HtrA1 protein production to be effectively reduced in osteogenic mASCs treated with siRNAs specific for *HtrA1* (Fig. 1A).

We next assessed the influence of loss-of-function of HtrA1 on the osteogenic potential of mASCs. mASCs treated with control siRNA underwent efficient osteoblastogenesis and mineralization following stimulation with osteogenic medium for 10 days, as determined by Alizarin red staining (Fig. 1B). However, Alizarin red staining of osteogenic-induced mASCs in which HtrA1 had previously been depleted was noticeably reduced (Fig. 1B). Similarly, HtrA1 deficiency also resulted in significant reductions in Alizarin red staining in mASCs induced to undergo osteogenic differentiation in response to BMP-2 treatment (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). Further quantitative analysis of extracted Alizarin red stain revealed the mineralizing capabilities of HtrA1-deficient mASC-derived osteoblasts to be significantly impaired ($P < 0.001$) compared with siControl (Fig. 1C).

In accordance with our previous findings [7], ATRA-mediated osteogenic induction of mASCs resulted in significant increases in both *HtrA1* (Fig. 1D) and *Alpl* (Fig. 1E) expression levels in a time-dependent manner. Similarly, ALP enzyme activity was also significantly enhanced in response to osteogenic induction at all time points tested (Fig. 1F). As expected, *HtrA1* knockdown of mASCs significantly suppressed *HtrA1* expression in osteogenic mASCs over the course of the study (Fig. 1D). ATRA-mediated increases in *Alpl* expression levels were also significantly impaired in HtrA1-deficient mASCs (Fig. 1E) and were accompanied by significant decreases in ALP

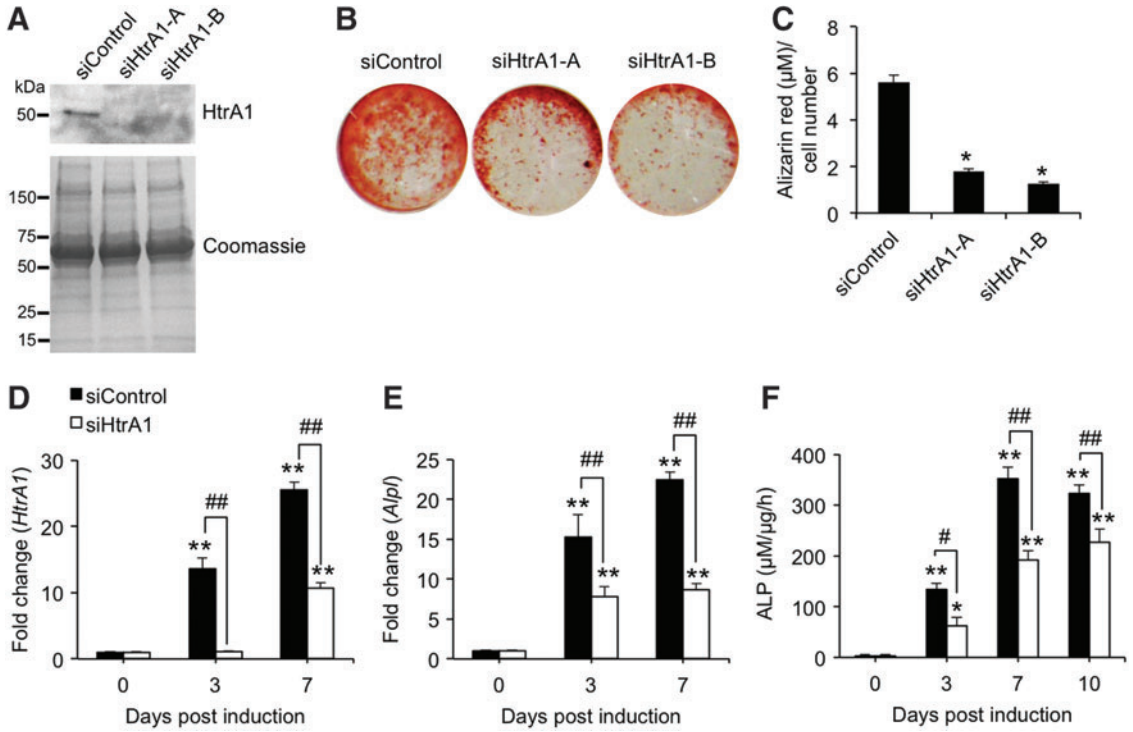

FIG. 1. Effect of *HtrA1* knockdown on osteogenic induction of mASCs. mASCs were pretreated with control siRNA (siControl) or two selected siRNAs targeting *HtrA1* (siHtrA1-A and siHtrA1-B) for 24 h and induced to undergo osteogenesis for up to 10 days. **(A)** Western blot analysis of HtrA1 protein in FCS-free concentrated supernatants from siRNA-treated mASCs after 4 days of osteogenic induction using an antibody specific for HtrA1. Gels were stained with Coomassie blue to confirm equal protein loading. **(B)** Alizarin red staining of siRNA-treated mASCs 10 days postosteogenic induction. **(C)** Extracted Alizarin red stain was quantified and normalized to cell number. $^{*}P < 0.001$ as compared to siControl using one-way ANOVA. Gene expression levels of *HtrA1* **(D)** and *Alpl* **(E)** were determined in osteogenic mASCs at selected time points using RT-qPCR and the fold change as compared to uninduced mASCs (day 0) determined using the $2^{-\Delta\Delta CT}$ method. **(F)** ALP enzymatic activity levels were measured in siRNA-treated mASCs over the course of 10 days of osteogenic differentiation using a colorimetric-based ALP activity assay. All values are expressed as mean ± SD (triplicates). $^{*}P < 0.05$, $^{**}P < 0.001$ as compared to uninduced mASCs at day 0; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ comparison between siControl and siHtrA1 using one-way ANOVA. ANOVA, analysis of variance; ALP, alkaline phosphatase; HtrA1, high temperature requirement protease A1; mASC, mouse adipose-derived stromal cells; RT-qPCR, quantitative reverse transcription PCR; siRNA, small interfering RNA. Color images available online at www.liebertpub.com/scd

enzymatic activity at all time points tested (Fig. 1F). These findings therefore demonstrate a functional role for HtrA1 in regulating ATRA-mediated mASC osteogenesis and in the generation of a mineralized matrix by mASC-derived osteoblasts.

HtrA1 deficiency impairs ATRA-mediated p70S6K activation in mASCs

Having demonstrated HtrA1 to be a necessary component for efficient mASC osteogenesis, we next considered its potential mode of action. Based on HtrA1's previously reported role in the activation of mTOR targets p70S6K and 4E-BP1 in tumor cell lines [16], we assessed the possible influence of HtrA1 deficiency on mTOR signaling in ATRA-stimulated mASCs. As no studies have yet sought to determine the effects of ATRA-mediated osteogenic induction on mTOR signaling in mASCs, we initially performed a series of western blot analyses to ascertain the activation status of kinases located both upstream and downstream of mTOR. Phosphorylation levels of Akt (Ser473), mTOR (Ser2448), p70S6K (Thr389), and 4E-BP1 (Thr37/46) were assessed in mASCs over the course of 2 h following ATRA-mediated osteogenic induction. A noticeable increase in Akt and p70S6K phosphorylation was already evident in osteogenic-induced mASCs after only 10 min and had reduced to basal levels by 1 h (Fig. 2A). However, basal levels of phosphorylated mTOR were only minimally affected after 10 min, and 4E-BP1 remained relatively unchanged at all time points tested. We next proceeded to investigate the influence of *HtrA1* silencing on Akt/mTOR/p70S6K/4E-BP1 phosphorylation in differentiating mASCs. Western blot analysis of cell lysates from HtrA1-deficient mASCs revealed no reduction in phospho-Akt levels, and only minor reductions in phospho-mTOR and–4EBP1 levels compared with siControl (Fig. 2B). By comparison, however, HtrA1-deficient mASCs demonstrated marked reductions in the levels of phosphorylated p70S6K and rpS6. These results are therefore suggestive of ATRA-mediated p70S6K activation as being a potential HtrA1 target and a means by which it could influence ATRA-dependent mASC osteogenesis.

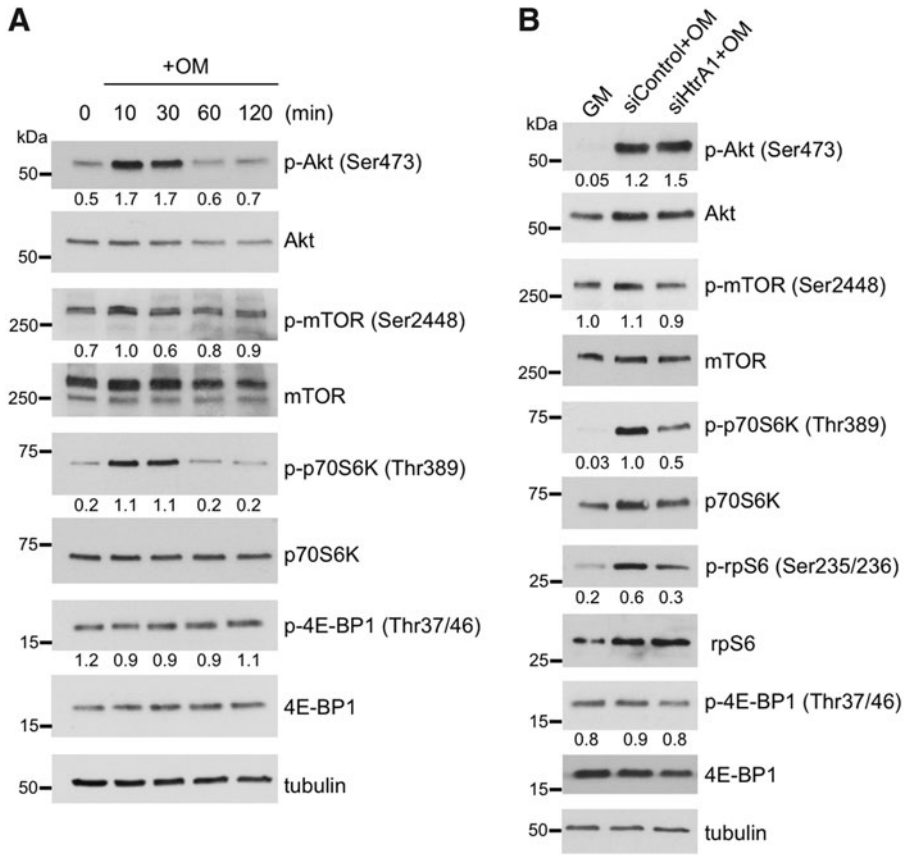

FIG. 2. Western blot analysis of kinase activity in mASCs in response to ATRA-mediated osteogenic induction. **(A)** mASCs were stimulated with OM for up to 2 h and cell lysates analyzed by immunoblotting at selected time points using antibodies specific for phosphorylated or nonphosphorylated Akt, mTOR, p70S6K, and 4E-BP1. A mouse anti-tubulin antibody was used to confirm equal protein loading. The influence of osteogenic induction on mASC protein phosphorylation was compared to undifferentiated mASCs (time point 0 min). **(B)** mASCs were pretreated with control siRNA (siControl) or siRNA targeting *HtrA1* (siHtrA1) for 24 h and stimulated with OM for 10 min. Untreated mASCs incubated in GM alone served as nondifferentiated controls. Cell lysates were extracted and analyzed by immunoblotting using antibodies specific for phosphorylated or nonphosphorylated Akt, mTOR, p70S6K, rpS6, or 4E-BP1. A mouse anti-tubulin antibody was used to confirm equal protein loading. Fold changes in phosphorylated protein levels versus nonphosphorylated protein levels are indicated. Results are representative of at least two separate experiments. 4E-BP1, eukaryotic initiation factor 4E binding protein 1; GM, growth medium; mTOR, mammalian target of rapamycin; OM, osteogenic medium.

Loss-of-function of p70S6K impairs mASC osteogenesis

To address the functional relevance of p70S6K activation in the context of mASC osteogenesis, we next assessed the effects of p70S6K inhibition on ALP expression and matrix mineralization in mASCs undergoing ATRA-mediated osteogenic differentiation. Short-term treatment of mASCs with the mTOR inhibitor rapamycin before osteogenic induction markedly reduced mTOR phosphorylation and completely abolished p70S6K phosphorylation, while Akt phosphorylation levels remained unaffected (Fig. 3A). Next, we evaluated the effects of long-term exposure of mASCs to rapamycin with regards to their ability to differentiate into mineralizing osteoblasts. Alizarin red staining of osteogenic mASCs was significantly reduced by rapamycin treatment in a concentration-dependent manner (Fig. 3B, C), thus confirming that mTOR signaling was required for mASC-derived osteoblastogenesis.

Further investigations employing siRNA-mediated knockdown of the p70S6K gene *Rps6kb1* were also performed to assess the effects of specifically inhibiting p70S6K activity on mASC osteogenesis. p70S6K protein and activity levels were noticeably reduced in *Rps6kb1*-deficient mASCs after short-term osteogenic induction (Fig. 4A). We next investigated the effects of *Rps6kb1* knockdown on mASC-derived osteoblast mineralization using Alizarin red staining. A marked reduction in Alizarin red staining was observed in *Rps6kb1*-deficient mASCs after 14 days of culture in osteogenic medium (Fig. 4B). Further quantitative analysis of extracted Alizarin red stain revealed the mineralizing capabilities of p70S6K-deficient mASC-derived osteoblasts to be significantly impaired compared with siControl ($P < 0.001$) (Fig. 4C). In support of these findings, significant reductions in Alizarin red staining of mASC-derived osteoblasts were also observed in cultures treated with the specific S6K1 inhibitor PF-4708671 (Supplementary Fig. S2). Gene expression analyses of mASCs undergoing osteogenesis confirmed

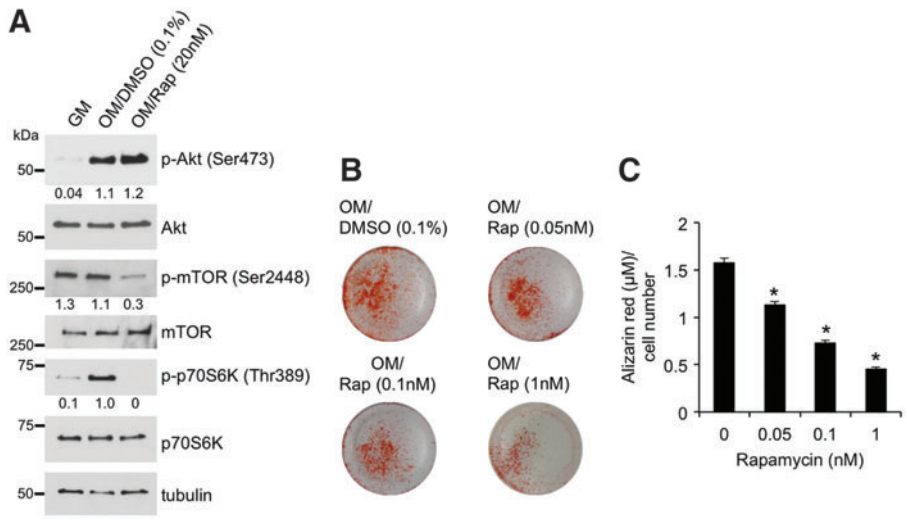

FIG. 3. Effect of rapamycin on osteogenic induction of mASCs. **(A)** mASCs were incubated with GM or OM for 10 min and cell lysates analyzed by immunoblotting using antibodies specific for phosphorylated or nonphosphorylated Akt, mTOR, or p70S6K. To assess the effects of mTOR inhibition on protein activation, cells were also pretreated for 2 h with 20 nM rapamycin (Rap) or vehicle (0.1% DMSO) before osteogenic induction. A mouse anti-tubulin antibody was used to confirm equal protein loading. Results are representative of at least two separate experiments. Fold changes in phosphorylated protein levels versus nonphosphorylated protein levels are indicated. **(B)** The influence of different concentrations of rapamycin on mASC-derived osteoblast mineralization was determined by Alizarin red staining at day 14. **(C)** Extracted Alizarin red stain was quantified and normalized to cell number and comparisons made between vehicle-treated and rapamycin-treated osteogenic mASCs. All values are expressed as mean ± SD (triplicates). $*P < 0.001$ as compared to mASCs treated without rapamycin using one-way ANOVA. Color images available online at www.liebertpub.com/scd

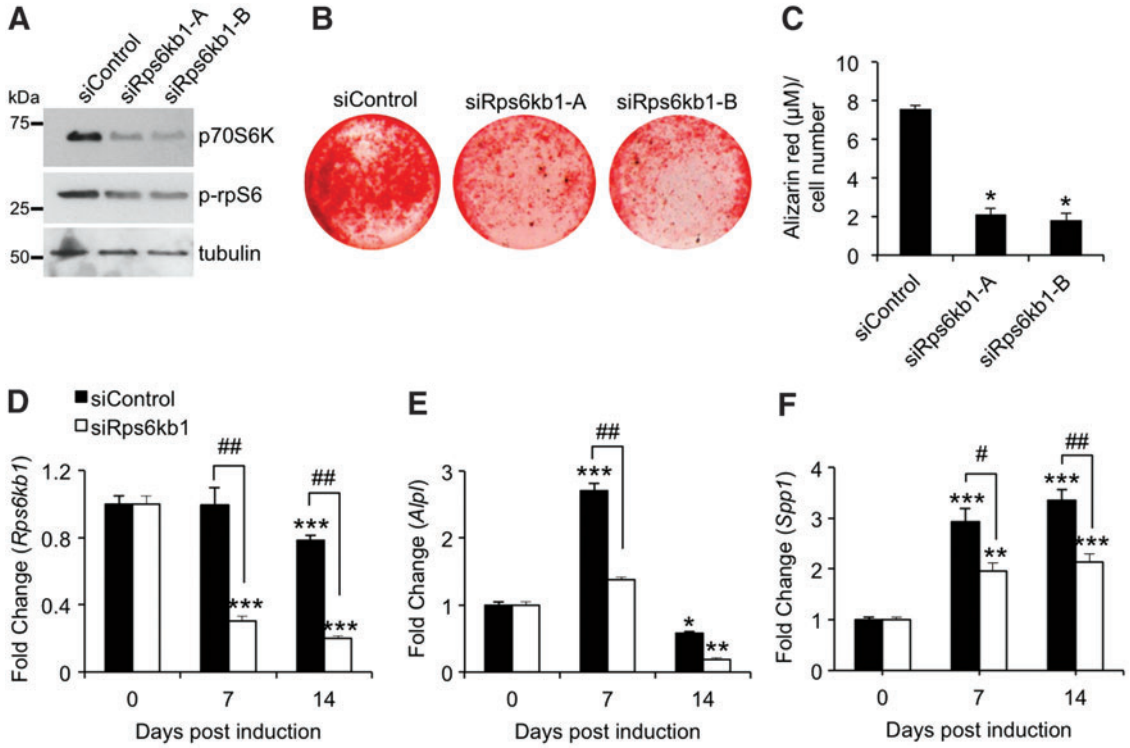

FIG. 4. Effect of *Rps6kb1* knockdown on osteogenic induction of mASCs. mASCs were pretreated with control siRNA (siControl) or two selected siRNAs targeting *Rps6kb1* (siRps6kb1-A and siRps6kb1-B) for 24 h and induced to undergo osteogenesis for up to 14 days. **(A)** Western blot analysis of p70S6K and phospho-rpS6 (p-rpS6) in siRNA-treated mASCs using antibodies specific for p70S6K and p-rpS6 respectively. A mouse anti-tubulin antibody was used to confirm equal protein loading. **(B)** Alizarin *red* staining of siRNA-treated mASCs 14 days postosteogenic induction. **(C)** Extracted Alizarin red stain was quantified and normalized to cell number. $*P < 0.001$ as compared to siControl using one-way ANOVA. **(D–F)** Gene expression levels of *Rps6kb1* **(D)**, *Alpl* **(E)**, and *Spp1* **(F)** were determined in osteogenic mASCs at selected time points using RT-qPCR and the fold change compared to uninduced mASCs determined using the $2^{-\Delta\Delta CT}$ method. All values are expressed as mean ± SD (triplicates). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to uninduced mASCs at day 0; $^{\#}P < 0.01$, $^{\#\#}P < 0.001$ comparison between siControl and siRps6kb1 using one-way ANOVA. Color images available online at www.liebertpub.com/scd

efficient *Rps6kb1* knockdown throughout the course of the study and revealed a significant reduction in *Rps6kb1* gene expression at day 14 in siControl in response to osteogenic induction (Fig. 4D). Furthermore, the expression levels of osteogenic markers *Alpl* (Fig. 4E) and osteopontin (*Spp1*) (Fig. 4F) in mASCs undergoing osteogenesis were significantly reduced in p70S6K-deficient cells. These results clearly identify p70S6K activation as being a necessary requirement for efficient osteogenic differentiation of mASCs and provide a potential means through which HtrA1 may regulate ATRA-mediated mASC osteogenesis.

Restoration of HtrA1-deficient mASC osteogenesis using a constitutively active p70S6K mutant

In view of the fact that mASC osteogenesis is dependent on both HtrA1 and p70S6K, and that loss-of-function of HtrA1 impairs p70S6K activation, we sought to determine whether transfection of mASCs with a constitutively active p70S6K mutant could relieve the detrimental effects of HtrA1 deficiencies on mASC osteogenesis. mASCs were transfected with either an empty plasmid, or plasmids encoding a rapamycin-resistant constitutively active (p70S6KCA) or kinase inactive (p70S6KKI) HA-tagged p70S6K [21]. The results shown in Fig. 5A are representative of the controls used to confirm that the plasmids encoding p70S6KCA and p70S6KKI were performing as expected. Both protein products were labeled with an HA-tag, and so plasmid-mediated protein expression could be accurately and specifically detected using an anti-HA antibody. As shown in the top lane of Fig. 5A, HA-labeled protein is evident in cells treated with plasmids encoding p70S6KCA and p70S6KKI as expected, but no signal is detected in cells treated with empty plasmid as no HA-labeled protein has been produced. Cells transfected with empty plasmid therefore serve as an additional control to confirm Western blot specificity and the robustness of the cell transfection system used.

The p70S6K substrate rpS6 is phosphorylated by activated p70S6K and therefore represents a useful means by which to visualize p70S6K activation by western blot. As such, inhibition of p70S6K activity by rapamycin treatment is expected to result in reduced phosphorylated rpS6 (p-

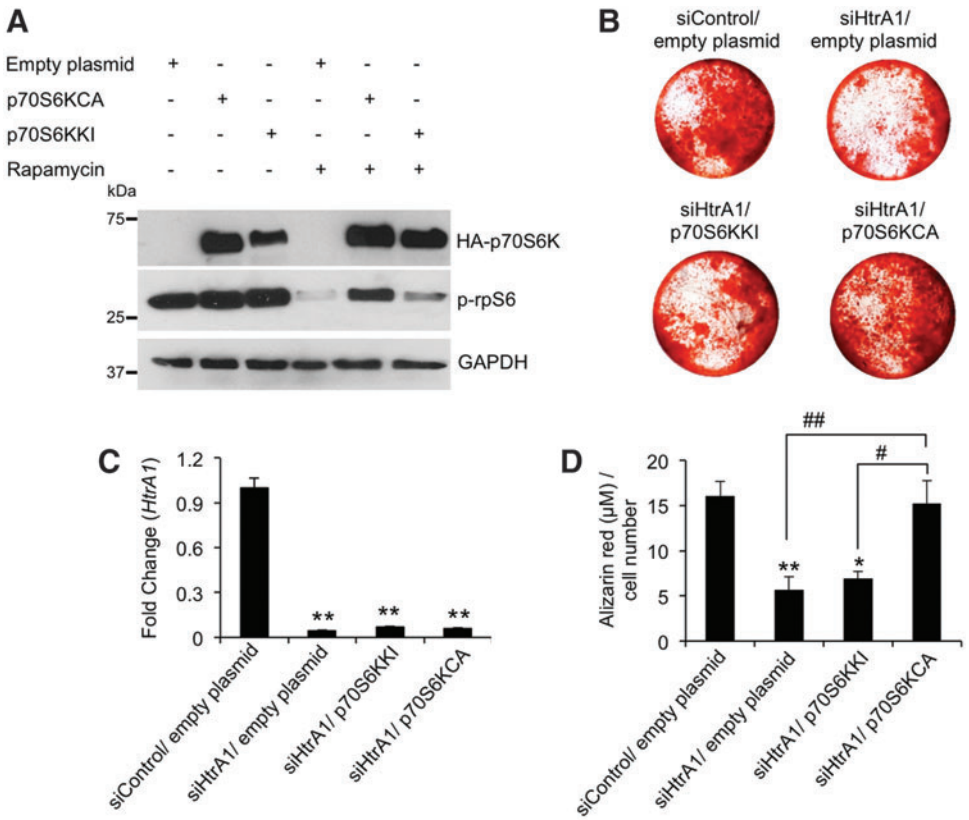

FIG. 5. Influence of p70S6K activity on siHtrA1-mediated suppression of mASC osteogenesis. **(A)** mASCs were transfected with empty plasmid, or plasmids encoding influenza hemagglutinin (HA)-tagged constitutively active (p70S6KCA) or kinase inactive (p70S6KKI) p70S6K mutants and immunoblotting performed after 24 h using an HA-probe antibody (HA-p70S6K) or anti-phospho-rpS6 antibody (p-rpS6). A mouse anti-GAPDH antibody was used to confirm equal protein loading. **(B)** The effect of plasmid DNA transfection on siRNA-mediated *HtrA1* gene silencing in mASCs was determined after 24 h using RT-qPCR. **(C)** The influence of plasmid DNA transfection on mASC-derived osteoblast formation in HtrA1-deficient mASCs was determined by Alizarin red staining at day 21 post osteogenic induction. **(D)** Extracted Alizarin red stain was quantified and normalized to cell number. All values are expressed as mean ± SD (triplicates). $^{*}P<0.01$, $^{**}P<0.001$ as compared to siControl/empty plasmid-treated cells; $^{\#}P<0.01$, $^{\#\#}P<0.001$ comparisons between siHtrA1/p70S6KCA and siHtrA1/p70S6KKI or siHtrA1/p70S6KCA and siHtrA1/empty plasmid using one-way ANOVA. Color images available online at www.liebertpub.com/scd

rpS6) levels. Indeed, upon rapamycin treatment of mASCs transfected with empty plasmid or plasmid encoding p70S6KKI, we observed a noticeable reduction in p-rpS6 levels in cells. This would be expected as neither the empty plasmid nor the kinase inactive p70S6KKI can generate active, rapamycin-resistant p70S6K. However, cells expressing the rapamycin-resistant, active P70S6KCA protein can still phosphorylate rpS6 even in the presence of rapamycin. Therefore, these results are confirmation that mASCs, when transfected with plasmid DNA, can produce the relevant p70S6K proteins and that they are either active (p70S6KCA) or inactive (p70S6KKI). Quantitative reverse transcription PCR confirmed that siRNA mediated reduction of *HtrA1* mRNA expression was unaffected in cells co-transfected with plasmid DNA (Fig. 5B).

Next, we transfected HtrA1-deficient mASCs with empty plasmid, p70S6KCA or p70S6KKI and assessed their ability to influence mASC-derived osteoblast formation through quantification of Alizarin red staining after 21 days. The assumption was that reduced p70S6K activity in HtrA1-deficient mASCs could be compensated for through expression of the constitutively active p70S6K (p70S6KCA), and thereby restore osteogenic potential. Indeed, as shown in Fig. 5C and D, Alizarin red staining was restored in HtrA1-deficient mASCs transfected with p70S6KCA. The kinase inactive (KI) p70S6K (p70S6KKI) was included to control as accurately as possible for the introduction of plasmid DNA into the cells and expression of p70S6K protein. It differs with respect to p70S6KCA in that it is not active, and so should not compensate for the reductions in p70S6K activity in HtrA1-deficient mASCs. This was indeed the case, as shown in Fig. 5C and D where Alizarin red staining could not be restored in HtrA1-deficient mASCs transfected with p70S6KKI. Empty plasmid was also included as an additional control, and it demonstrated no influence over mASC osteogenesis as expected. Taken together, these findings identify p70S6K as being of critical importance in ATRA-mediated mASC osteogenesis and that activation of p70S6K is reliant, at least in part, on the actions of HtrA1.

Discussion

mASCs represent a readily available source of osteoprogenitor cells, which unlike mBMSCs, have the advantage of being able to sustain a high level of osteogenic differentiation potential with age and under conditions of low bone quality [4, 22–25]. Subsequently, mASCs are fast becoming the preferred choice for stem cell-based approaches in bone tissue engineering [26,27]. Certainly, results from our previous studies have confirmed that mASCs harvested from SAMP6 mice, a model for senile osteoporosis, have the capability of increasing bone quality when re-injected back into SAMP6 tibia [5]. However, despite their widespread usage, the underlying mechanisms through which mASC osteogenic differentiation is controlled remains incompletely understood.

In the current report, we identify the serine protease HtrA1 as being a positive regulator of ATRA-induced mASC osteogenesis and mASC-derived osteoblast mineralization. Furthermore, we provide evidence, which supports p70S6K as playing a role in mediating the pro-osteogenic effects of HtrA1 in mASCs in response to ATRA.

Mammalian HtrA1 was originally identified by Zumbrunn and Trueb [28] and has since been implicated in numerous biological processes and diseases [15,29]. Findings from our own studies have revealed HtrA1 to be a potent modulator of hBMSC multipotency as evidenced by its ability to inhibit adipogenesis and stimulate osteogenesis [7]. However, several studies also exist in which HtrA1 has been classified as a negative regulator of osteogenesis both in vitro and in vivo [30,31]. Clearly therefore, further investigations are required to clarify HtrA1's function in osteogenesis and to ascertain its potential mechanism of action. Here, we provide further evidence in support of HtrA1's role as a positive regulator of multipotent stromal cell osteogenesis.

HtrA1 is classified as a secreted serine protease and as such, its influence over cellular processes is largely thought to be due to its extracellular actions [7,20,32]. However, it is also equally likely that HtrA1 instigates many of its effects intracellularly. Indeed, HtrA1 has been shown to interact with and functionally regulate several intracellular substrates including tubulin [33], proTGFβ1 [34], tau [35], X-linked inhibitor of apoptosis protein (XIAP) [36], and TSC2 [16]. In the context of the present study, HtrA1's regulatory influence over TSC2 activity holds particular relevance given the importance of mTOR signaling in stem cell multipotency [17–19].

The mTOR protein makes up the catalytic subunit of two separate complexes, namely mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [37]. mTORC1 functions to control cell growth and protein synthesis through phosphorylation of 4E-BP1 and p70S6K, and it has been implicated in osteoblast differentiation [17,38–40]. mTORC1 is negatively regulated by the GTPase-activating protein TSC1/2 complex and as such, relies on the actions of Akt for its activation through phosphorylation of TSC2 [41]. Although several studies have demonstrated activation of the Akt/mTOR signaling pathway in response to ATRA [42–44], no investigations have yet been undertaken to examine Akt/mTOR activation in ATRA-stimulated mASCs or to evaluate its consequences for their commitment toward osteoblasts.

Our findings have demonstrated that the Akt/mTOR/p70S6K pathway is rapidly activated in mASCs in response to ATRA. Similar rapid increases in several other kinase cascades have previously been demonstrated in various cell systems in response to ATRA [45–48]. Such effects are considered to be independent of the classical genomic effects of ATRA, and these are instead regulated through atypical, nongenomic events possibly through interactions with membrane-associated retinoic acid receptors [49]. The ability of the mTOR inhibitor rapamycin to completely suppress ATRA-mediated p70S6K activation in these cells suggests that despite the minimal increases in phospho-mTOR (Ser2448), ATRA-mediated p70S6K activation in mASCs is rapamycin sensitive and as such, reliant on mTOR signaling.

It is important to note that although p70S6K is well recognized as a downstream target of mTOR, phosphorylation of mTOR at Ser2448 is also considered to represent a feedback signal from p70S6K [50]. If indeed the case, then ATRA-mediated p70S6K activation in mASCs may possibly be reliant on increases in mTOR activity through the phosphorylation of sites other than Ser2448. Certainly,

mTOR has been reported to have several potential phosphorylation sites whose functions remain largely undefined [51]. As with rapamycin treatment, loss-of-function of HtrA1 also resulted in reductions in mTOR and p70S6K phosphorylation in the absence of any changes in phospho-Akt. However, in contrast to rapamycin, the removal of HtrA1 was unable to completely abolish mTOR or p70S6K phosphorylation.

Although p70S6K activity is considered to be of paramount importance in determining mASC adipocyte lineage commitment [52], its functional role in mASC osteogenesis has not yet been established. Our findings from studies using the mTOR inhibitor rapamycin, along with siRNA-dependent inhibition of *Rps6kb1* gene expression, confirmed that the mTOR/p70S6K signaling pathway was indeed an essential requirement for efficient mASC osteogenesis and mASC-derived osteoblast mineralization. As far as we are aware, this is the first report to demonstrate such a role for mTOR/p70S6K in ATRA-mediated mASC osteogenesis. Therefore, these studies identified both HtrA1 and mTOR/p70S6K as being important regulators of ATRA-mediated mASC osteogenesis.

However, it was still unclear as to whether HtrA1's ability to regulate p70S6K phosphorylation in response to ATRA was directly related to its pro-osteogenic effects. We therefore performed a study in which we introduced plasmids encoding DNA for either constitutively active or kinase inactive mutants of p70S6K into HtrA1-deficient mASCs in an attempt to rescue osteoblastogenesis. Indeed, our results revealed that the mineralizing capacity of HtrA1-deficient mASC-derived osteoblasts could be fully restored when cells were engineered to express the constitutively active p70S6K mutant.

These findings therefore identify p70S6K activation as a regulatory target of HtrA1 and an important event in mediating HtrA1's pro-osteogenic effects in ATRA-stimulated mASCs. However, based on the fact that mTOR phosphorylation at Ser2448 may be the result of feedback regulation by p70S6K, further investigations are needed to determine whether HtrA1 can directly influence mTOR activation. Certainly, we would anticipate that if HtrA1 were acting to regulate p70S6K activity through its interaction with TSC2 [16], then reductions in mTOR activity would be apparent [53]. However, the suggestion that the TSC-complex may in fact regulate p70S6K independently of mTOR [54], may offer an additional means by which HtrA1 could activate p70S6K without the need for alterations in mTOR activity.

Alternatively, HtrA1 may act to regulate p70S6K phosphorylation through mTOR, but in a TSC2-independent manner. Certainly, mTOR is not solely reliant on TSC2 inhibition for its activation as confirmed by studies in which Akt was shown to activate mTOR by relieving the inhibitory effects of proline-rich Akt/PKB substrate 40 kDa (PRAS40) on mTORC1 [55], and more recently, through its ability to promote mTORC1 phosphorylation at Ser1415 via the actions of IkB kinase alpha (IKKα) [56]. Further studies are therefore required to ascertain the involvement of TSC2 and mTOR in mediating the effects of HtrA1 on p70S6K phosphorylation in osteogenic mASCs.

Despite us having now demonstrated HtrA1 to be a positive regulator of both mASC and hBMSC [7] osteogenesis, its role in mediating bone formation remains highly controversial. This is highlighted by findings from previous studies in which HtrA1 was deemed to be a negative regulator of osteogenesis in stromal cells derived from long-term bone marrow cultures [31] and mouse 2T3 osteoblasts [57]. By contrast however, a more recent report has identified HtrA1 as being a necessary requirement for the osteogenic differentiation of periodontal ligament cells [58]. One possible explanation for such discrepancies may lie in the fact that in each of these studies, a different cell culture system was used. If indeed the case, this would imply that HtrA1 acts to mediate cell-specific responses to osteogenic stimuli, the result of which may impart an inhibitory or enhancing effect on osteogenic induction.

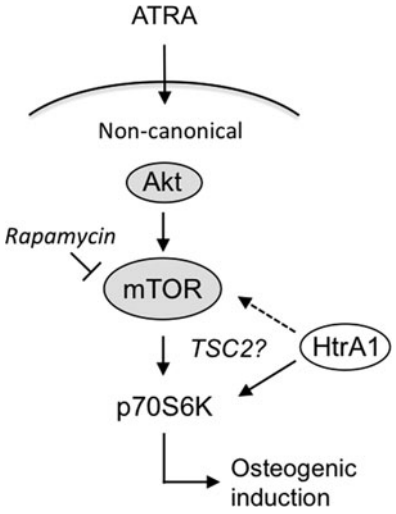

FIG. 6. Model of ATRA-induced mASC osteogenesis. Based on our findings, we propose that ATRA drives osteoblast commitment of mASCs through activation of p70S6K in a noncanonical and nongenomic manner. This appears not only to be dependent on rapamycin-sensitive signaling pathways, but also on the actions of HtrA1. However, further investigations are required to determine HtrA1's mechanism of action and to clarify the potential involvement of the HtrA1 substrate TSC2 in mediating its effects on p70S6K activity and mASC osteogenesis. ATRA, all-*trans* retinoic acid; TSC2, tuberous sclerosis complex 2.

Interestingly, our new findings also demonstrate that HtrA1's involvement in regulating mASC osteogenesis extends beyond it's ability to influence ATRA-mediated osteogenic induction as evidenced by significant reductions in mineral formation in HtrA1-deficient mASC cultures stimulated with BMP-2.

To try and further elucidate HtrA1's role in regulating bone formation, investigations have also been conducted in mice with a targeted gene deletion of *HtrA1* [30]. However, although HtrA1-deficient mice displayed significant increases in several bone parameters, the influence of such changes on bone quality and strength remain to be determined. Furthermore, no studies were undertaken to address the possible involvement of compensatory mechanisms in HtrA1-deficient mice, such as the upregulation of other members of the HtrA family (eg, HtrA3 and HtrA4), which may also have an influence on bone formation. Clearly,

more in-depth investigations are required to reconcile these conflicting studies and thereby help clarify HtrA1's role as a modulator of bone formation.

In summary, we have identified p70S6K as an important regulator of mASC osteogenesis, being activated in response to ATRA via pathways involving mTOR and HtrA1 (Fig. 6). As such, it is proposed that HtrA1 represents a newly identified positive regulator of ATRA-mediated mASC osteogenesis and mASC-derived osteoblast mineralization.

Acknowledgments

SG and GF were supported by SNSF grants 31003A_134935 and 31003A_156313. AM and ANT were supported by the Uniscientia Foundation and Forschungskredit University of Zurich. CLF was supported by the Whitaker International Program.

Author Disclosure Statement

The authors declare that they have no competing financial interests.

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Received for publication December 3, 2015
Accepted after revision March 4, 2016
Prepublished on Liebert Instant Online March 7, 2016

4. Unpublished data

4.1 Osteogenic differentiation of MSCs from wild-type (WT) and *Htra1*-knockout (*Htra1*-KO) mice

In order to examine the influence of HTRA1 loss on MSC differentiation, we harvested adipose-derived stromal cells (ASCs) and bone marrow stromal cells (BMSCs) from WT and *Htra1*-KO mice, and induced them towards osteoblasts using osteogenic induction media specific to each cell type. ASCs were incubated for up to 19 days with DMEM low glucose supplemented with 10% FCS, 10 mM β-glycerophosphate, 50 μm ascorbic acid and 5 μM retinoic acid. BMSCs were incubated for up to 21 days in alpha MEM supplemented with 10% FCS, 10 mM β-glycerophosphate, 50 μm ascorbic acid and 100 nM dexamethasone. In each case, osteoblast-mediated matrix mineralization was determined using Alizarin Red S staining at specific time points. RT-qPCR analysis of *Htra1*, *Htra3* and alkaline phosphatase (*Alpl*) gene expression was also performed in ASCs at specific time points. As expected, *Htra1* expression levels were significantly reduced in ASCs from *Htra1*-KO mice at all time points following osteogenic induction as compared to WT-derived ASCs (Figure 12A). However, *Htra3* expression levels remained comparable between ASCs from WT and *Htra1*-KO mice, and were shown to decrease in response to osteogenic induction. Comparisons of *Alpl* expression levels between WT and *Htra1*-KO mice showed only minor deviations, with a significant increase being observed in ASCs from WT mice at day 3 only. Similarly, the ability of ASCs to generate osteoblast-derived mineralized matrix was comparable between WT (N=2) and *Htra1*-KO (N=2) mice (Figure 12B). This was also apparent when comparing the osteogenic capacity of BMSCs from WT (N=3) and *Htra1*-KO (N=3) mice, where Alizarin Red S staining was observed at equivalent levels in both mouse strains (Figure 13). These findings therefore indicate that osteogenic differentiation is unaffected in MSCs derived from HTRA1-deficient mice, and are consistent with the lack of a bone phenotype in *Htra1*-KO mice (Result section; 3.1.1).

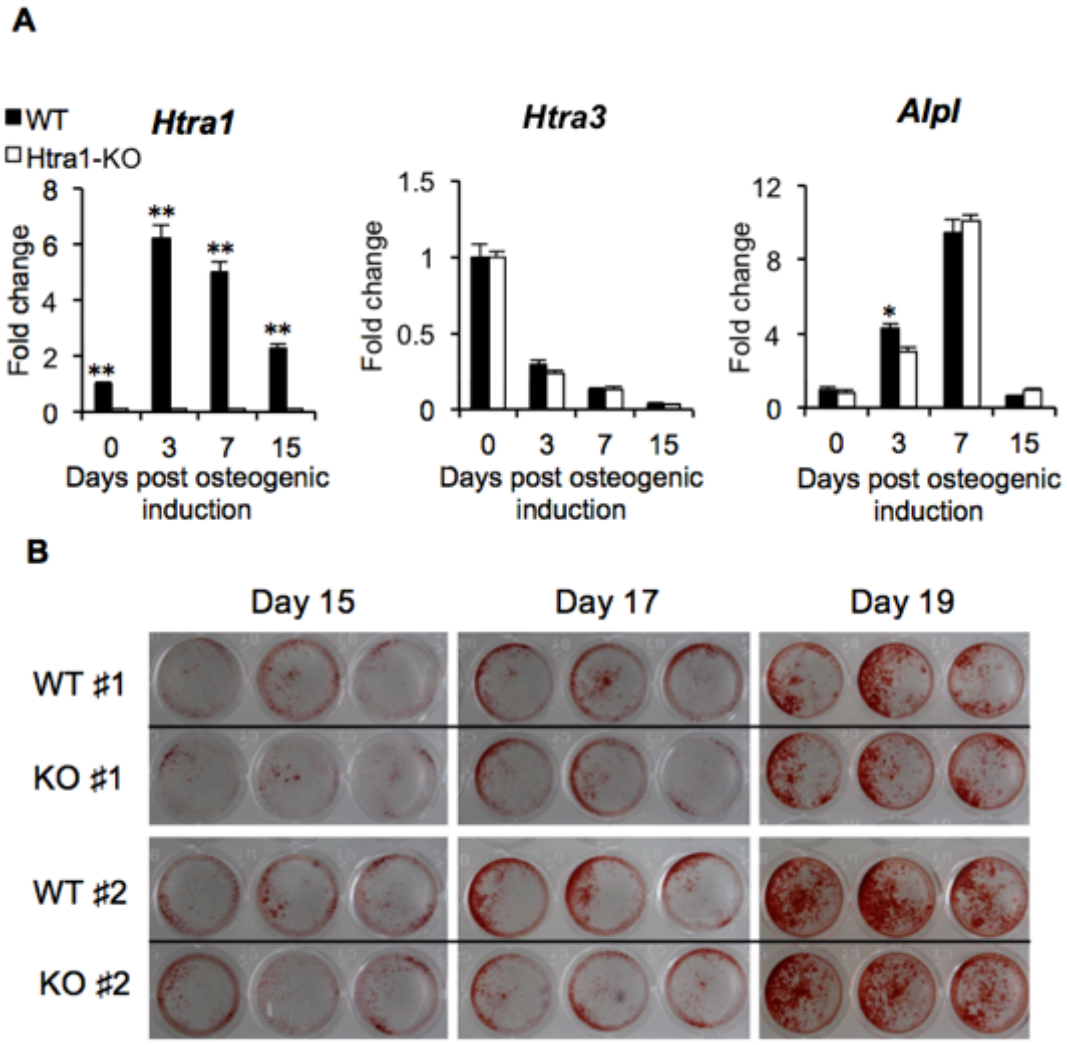

Figure 12: Osteogenic differentiation of ASCs from WT and *Htra1*-KO mice
ASCs isolated from wild-type (WT; N=2) and *Htra1*-knockout (KO; N=2) mice were incubated in osteogenic medium for up to 19 days and gene expression of *Htra1*, *Htra3* and *Alpl* determined by qPCR (**A**), and matrix mineralization determined by Alizarin Red S staining (**B**). * $P<0.01$, ** $P<0.001$ as determined by ANOVA. All tests were conducted in triplicates and values presented as mean ± S.D.

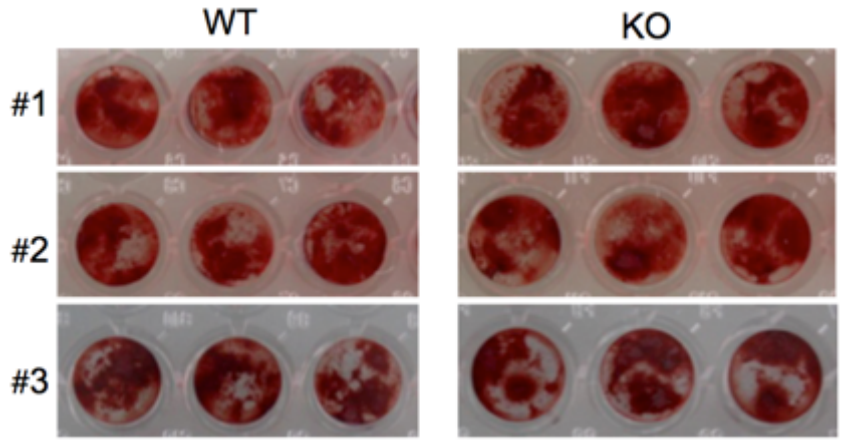

Figure 13: Osteogenic differentiation of BMSCs from WT and *Htra1*-KO mice
BMSCs isolated from wild-type (WT; N=3) and *Htra1*-knockout (KO; N=3) mice were incubated in osteogenic medium for up to 21 days and matrix mineralization determined by Alizarin red S staining.

4.2 Immunohistological analysis of MMP-13 in tissue sections of the osteotomy site from wild-type (WT) and *Htra1*-knockout (*Htra1*-KO) mice

Based on the fact *Mmp13* expression was so dramatically increased in *Htra1*-deficient C3H10T1/2 cells (Result section; 3.1.1), we also compared MMP-13 protein levels in the paraffin sections of the femoral osteotomy sites of both strains. Comparable levels of MMP-13 protein were detected in the cartilaginous callus of WT (Figure 14A, C) and *Htra1*-KO mice (Figure 14B, D) at day 21 post-surgery, primarily localized to hypertrophic chondrocytes. Therefore, despite HTRA1 loss having a dramatic effect on *Mmp13* expression *in vitro*, this effect could not be reproduced *in vivo*.

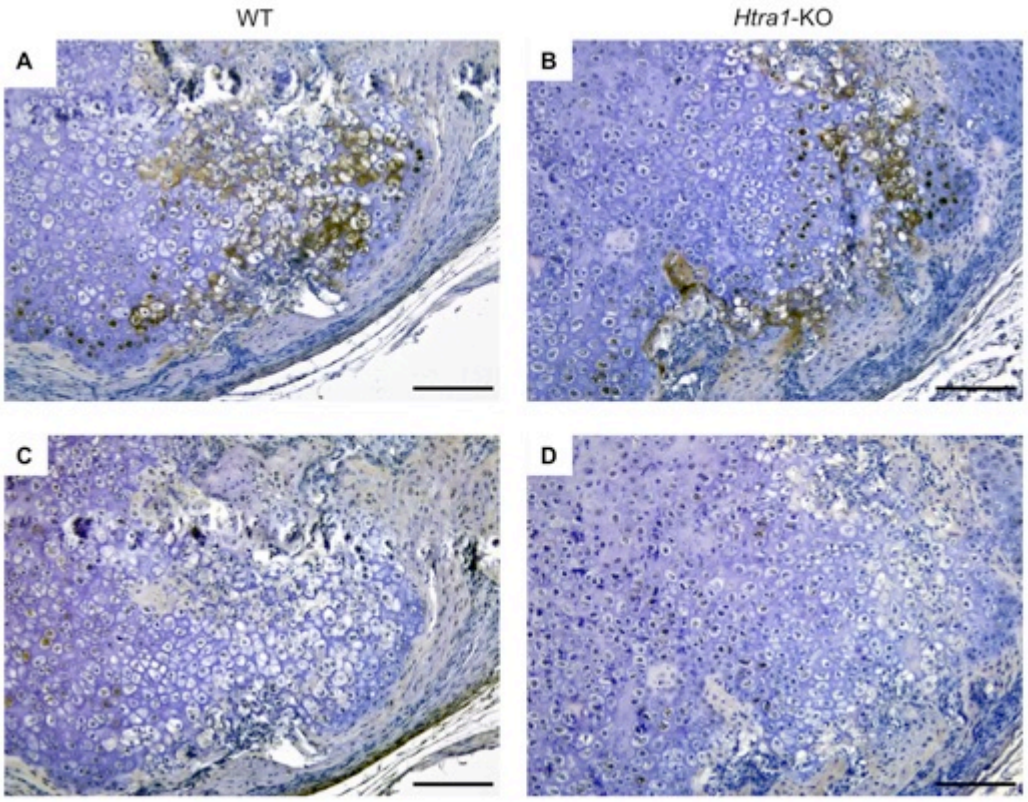

Figure 14: Immunostaining of MMP-13 in callus tissue
Representative micrographs of anti-MMP-13 (*A*, *B*), or normal rabbit serum (*C*, *D*) stained paraffin wax sections of femurs from WT (*A*, *C*) and Htra1-KO (*B*, *D*) mice 14 days after osteotomy. MMP-13 staining was detected using horseradish peroxidase-diaminobenzidine (*brown*) and sections counterstained with Harris modified haematoxylin (*blue*). Scale bar = 100 μm.

4.3 Biomechanical analysis of femurs from 52-week-old wild-type (WT) and *Htra1*-knockout (*Htra1*-KO) mice

The aim of this experiment was to compare the mechanical properties of mouse femurs from WT and *Htra1*-KO mice, by conducting a three-point flexure test. Dependent variables, extracted to assess mechanical properties, included force and displacement at ultimate, yield and break strength, as well as the post-yield displacement, stiffness, and total and elastic toughness. Femur length, diameter and cross section were measured using Vernier callipers. A three-point bending test was then conducted by placing the femora between two supports 6 mm apart, and positioning the loading pin 55% of the length from the proximal side. Testing was carried out on a Zwick Z005 (Zwick-Roell, Ulm Germany) fitted with a 5 kN load cell. A 1-N preload oriented the femur so that the load was applied in the anterior-posterior direction and load-displacement values were recorded at a crosshead speed of 0.5 mms-1 until failure. From this data, ultimate, yield and

break point, as well as post-yield displacement, stiffness, and total and elastic toughness could be derived. The results of the independent t-test suggest that loss of HTRA1 had insubstantial effects on almost all measured mechanical properties (Table 1). However, a significant difference was observed between the two groups for stiffness ($p < 0.05$) where the *Htra1*-KO mice showed a higher mean value (0.260 Nμm-1 c.f. 0.222 Nμm-1). Undoubtedly, the power of this analysis would have been enhanced had more samples been available for testing. Therefore, future studies using larger animal cohorts may be warranted, and would help ascertain whether bone quality is indeed improved in HTRA1-deficient mice.

Mechanical property	**Wild-type (N = 6)**		***Htra1*-KO (N = 5)**		**P-value**
	Mean	**S.D.**	**Mean**	**S.D.**	
Ultimate strength (N)	22.111	3.158	23.716	2.763	0.398
Ultimate strength displacement (μm)	186.418	32.403	179.190	66.597	0.819
Yield strength (N)	16.542	4.478	18.332	4.310	0.519
Yield displacement (μm)	92.974	38.247	73.952	20.870	0.348
Post-yield displacement (μm)	159.078	83.635	267.391	181.524	0.221
Break force (N)	21.144	4.227	21.872	1.994	0.733
Break displacement (μm)	252.052	105.514	341.344	167.325	0.308
Stiffness (Nμm_-1)	0.222	0.0256	0.260	0.0126	0.015*
Total toughness (J)	4.051	1.702	6.579	3.786	0.174
Elastic toughness (kJ)	1.079	0.652	0.947	0.441	0.712

Table 1: Values for the mechanical properties of wild-type and *Htra1*-KO groups, and testing for comparison of means. P-values were determined using Student's t-test.

4.4. Effect of *Htra1* knockdown on mineralized matrix production in ATDC5 cells cultures.

The influence of HTRA1 loss on osteogenesis was also evaluated in the pre-chondrocyte ATDC5 cell line. ATDC5 cells were transduced with a non-target control shRNA construct (shControl) or specific for *Htra1* (sh*Htra1*^{84} or

sh*Htra1*^{86}), and following puromycin selection, were incubated with DMEM-F12 supplemented with 10 µg/mL ITS for one week to induce chondrogenesis. Osteogenesis was then subsequently induced using αMEM culture medium supplemented with 10 µg/mL ITS and 10 mM β-glycerophosphate. Osteoblast-mediated matrix mineralization was assessed at day 3, 4 and 6 following osteogenic stimulation using Alizarin Red S stain. RT-qPCR analysis confirmed that *Htra1* expression levels were significantly reduced in both sh*Htra1*^{84} and sh*Htra1*^{86} treated cultures at day 3 and 4 after osteogenic induction as compared to the shControl. However, by day 6, *Htra1* expression levels had partly recovered in sh*Htra1*^{84}-treated ATDC5 cells, and were completely restored in sh*Htra1*^{86}-treated ATDC5 cells (Figure 15A). HTRA1-deficient ATDC5 cell cultures exhibited a delay in matrix mineralization as demonstrated by a marked reduction in Alizarin Red S staining at all time points tested (Figure 15B).

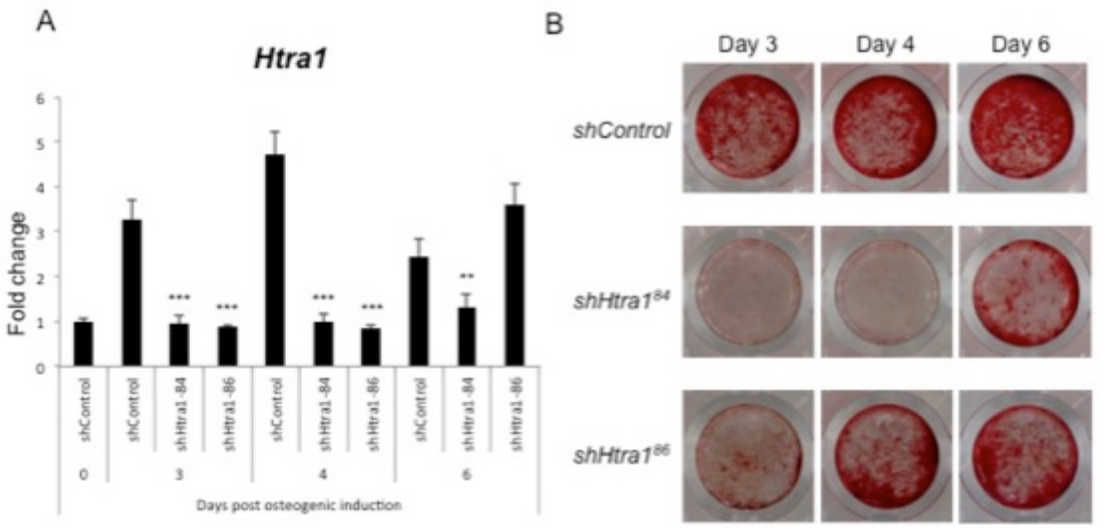

Figure 15: Osteogenic differentiation of ATDC5 cells.
ATDC5 cells were incubated in chondrogenic media for 7 days followed by osteogenic induction for 6 additional days. Gene expression of Htra1 was determined in ATDC5 cells transduced with non-target control shRNA (shControl) or Htra1-specific shRNA (sh*Htra1*^{84} or sh*Htra1*^{86}) by RT-qPCR (A), and matrix mineralization determined by Alizarin Red S staining (B). *** P<0.01 and ** P<0.05, as determined by ANOVA compared to shControl for each time point. All tests were conducted in triplicates and values presented as mean ± S.D.

5. General Discussion and Perspectives

HTRA1 is a diverse protease, having been associated with numerous biological processes [225]. Subsequently, it is considered to play an important role in a number of pathological disorders, including musculoskeletal disease (MSD) [222]. However, despite the fact that many studies have identified HTRA1 in calcified tissue, its actual role in bone formation and repair remains an enigma.

This study aims at improving our understanding of the role of HTRA1 in bone formation and regeneration using both an *in vitro* endochondral model and an *in vivo* femur osteotomy model. We demonstrated that *Htra1* silencing in C3H10T1/2 cells enhanced the expression of several osteogenic markers, accelerated matrix mineralization, and resulted in decreased chondrogenic marker expression. Although our findings showed that HTRA1 deficiency had a profound effect on osteogenic differentiation *in vitro*, they failed to confirm that bone development was affected in 16-week-old HTRA1-deficient mice. Interestingly, however, significant improvements in various structural bone parameters were observed in 52-week-old HTRA1-deficient mice as compared to age-matched wild-type controls, thereby providing a possible link between HTRA1 production/activity and age-related bone loss.

Indications that HTRA1 may play a role in regulating bone formation initially came from developmental studies performed in mice, where *in situ* hybridisation and immunohistochemical analyses identified HTRA1 in the bone matrix of adult bones [233,295]. Additional studies performed on embryonic mice further demonstrated the presence of HTRA1 in developing bone tissue, and further identified HTRA3 in comparable locations [227]. However, it was not until several years later that evidence first emerged of a possible functional role for HTRA1 in bone formation.

Although HTRA1's influence on osteogenic differentiation has been extensively investigated, its role in osteogenesis remains highly disputed as highlighted by the increasing number of published articles currently available [93,227,233,262,295,298-301,303,304]. HTRA1 has been shown to act as a negative regulator of mouse 2T3 osteoblasts BMP-2-induced osteogenesis [93]. It was demonstrated that although osteogenic induction of mouse 2T3 osteoblasts with BMP-2 increased *Htra1* expression levels, cultures treated with exogenous recombinant HTRA1 protein failed to mineralize [93]. Furthermore,

Htra1 gene silencing using siRNA specific to *Htra1* promoted calcium deposition and subsequent matrix mineralization of the 2T3 cells. In support of this, Wu *et al.* (2013) [304] confirmed the detrimental role of HTRA1 on osteogenic differentiation using the osteoblastic mesenchymal cell line KusaO. The authors observed an increase in HTRA1 protein secretion when osteoclasts were stimulated with RANKL, which resulted in the inhibition of osteoblast differentiation of the long-term bone marrow KusaO cell cultures. Additionally, recombinant HTRA1 treatment led to decreases in the expression levels of *Alp, Runx2, Ocn and Opn* in BMP-2 stimulated KusaO cells [304]. Furthermore, this was accompanied by a decrease in BMP2-induced Smad1/5/8, ERK1/2 and p38 activation, implicating HTRA1 in the regulation of BMP2 signalling. In addition, levels of osteogenic markers *Runx2*, connective tissue growth factor (*Ctgf*) and plasminogen activator inhibitor 1 (*Pai1*) were increased in TGF-β stimulated mouse embryonic fibroblasts (MEFs) isolated from *Htra1*-knockout mice as compared to wild-type mice [262]. Finally, the observation that HTRA1-deficient mice have a moderately improved bone phenotype [262], suggests that HTRA1's effect on bone physiology might go beyond simply affecting mineral formation *in vitro*.

However, an equal number of published studies are now challenging this concept of HTRA1 as a negative regulator of osteogenic lineage commitment. Several research groups have reported that loss-of-function of HTRA1 in primary human and mouse MSCs results in impaired osteogenic differentiation [298,300,301]. Studies conducted in our laboratory demonstrated that HTRA1 acted as a positive regulator of hBMSC and mASC osteogenic differentiation [298,300]. Matrix mineralization by hBMSC-derived osteoblasts was impaired following siRNA-mediated *HTRA1* gene silencing, and was enhanced by the addition of exogenous HTRA1 protein [298]. These findings are supported by other investigations using human periodontal ligament cells (hPDLCs) [301], in which matrix mineralization was also significantly reduced following loss-of-function of HTRA1. In addition, Glanz *et al.* (2016) [300] demonstrated that all-*trans* retinoic acid (ATRA)-induced matrix mineralization was impaired in HTRA1-deficient mASCs, and that these effects were associated with alterations in mTOR signalling.

The lack of consistency among these *in vitro* studies regarding the role of HTRA1 in osteogenesis may be explained by deviations in the various sources of cells

used. Indeed, studies in which HTRA1 was deemed a positive regulator of osteogenesis were conducted using primary cells (i.e. human and mouse MSCs). By contrast, studies in which HTRA1 was implicated as a negative regulator of osteogenesis principally used immortalised cell lines such as 2T3 osteoblasts or KusaO cells. In all cases, HTRA1's potential to influence osteogenesis *in vitro* was investigated in the context of it having a direct effect on the cell's ability to develop into bone-forming osteoblasts. However, it has previously been reported that HTRA1 is differentially expressed during MSC condensation and subsequent bone formation, where it was identified primarily in hypertrophic chondrocytes at the commencement of mineralization [295].

Intramembranous and endochondral ossification are two mechanisms that lead to bone formation, but they occur at different stages of development and are specifically regulated [36]. Therefore it is conceivable that HTRA1's potential to modify osteogenic differentiation and bone mineral formation may be determined not only by the cell-type, but also by the stage of bone development [306]. This may therefore offer an additional explanation for the contrasting effects of HTRA1 deficiency on ATDC5- and C3H10T1/2-mediated matrix mineralization (unpublished results 4.4).

Another point to consider is the concentration range of HTRA1, which might influence its mode of action. Indeed, when HTRA1 was reported as having a negative effect on KusaO cells [304], cultures had been treated with relatively low concentrations of recombinant protein, while its positive effect on hBMSC osteogenesis was observed when high concentrations of recombinant HTRA1 were used [298]. It's conceivable that HTRA1 acts in a dose-dependent manner and this effect combined with differences in protein production resulted in differential effects on lineage commitment.

In the current report, we have extended these investigations to include the multipotent murine mesenchymal stem cell line C3H10T1/2. The C3H10T1/2 cell line was established in 1973 from the isolation and derivation of C3H mice embryos [307]. C3H10T1/2 cells exhibit a stable fibroblast-like phenotype in a non-confluent culture; they are sensitive to contact inhibition; and are hypertetraploids, since they carry 81 chromosomes [308]. They were originally used as a model to study oncogenic transformation and cell synchronisation [307]. Furthermore, they remain negative for C-type retrovirus expression even after prolonged passages, and are non-tumorigenic [308]. Their ability to

differentiate into specific cell types was a fortuitous discovery. Constantinides *et al.* (1977) [309] investigated the effect of the chemotherapeutic agent 5-azacytidine (5-Aza-CR) on the malignant transformation of C3H10T1/2 cells. The researchers noticed that C3H10T1/2 cultures exhibited multinucleated and elongated cultures with myotube-like morphology [309]. A year later, it was demonstrated that these differentiated C3H10T1/2 cells were able to contract under acetylcholine treatment and expressed a calcium dependent ATPase, myosin [310]. Other studies addressed the question whether C3H10T1/2 cells were able to generate other mesodermal lineages such as adipocytes, osteoblasts and chondrocytes, since these cell types share the same embryonic origin as myocytes. Taylor and Jones (1979) [311] treated C3H10T1/2 cells with 5-Aza-CR and observed that in addition to myocytes, C3H10T1/2 cells could differentiate into adipocytes as well as chondrocytes and this potential for tri-lineage commitment was regulated by temporal changes rather than a concentration gradient. Furthermore, it is known that BMPs are crucial factors in cartilage and bone production, and it was shown that under BMP-2 induction, C3H10T1/2 cells displayed enhanced osteogenic potential which appeared to be closely related to adipogenic differentiation. Multilineage capacities of the C3H10T1/2 cells have subsequently led to their incorporation in studies examining MSC lineage commitment [312-314]. We confirmed that C3H10T1/2 cells were capable of undergoing chondrogenic and osteogenic differentiation in response to BMP-2, and that this was accompanied by temporal increases in *Htra1*-expression levels. However, in contrast to our previous studies using primary MSCs [298], *Htra1* knockdown had a positive impact on C3H10T1/2-mediated matrix mineralization. This, together with the observed reductions in chondrocyte-associated differentiation markers, provided strong evidence to support a role for HTRA1 in regulating the osteochondral differentiation of C3H10T1/2 cells in response to BMP-2. These findings also appeared to strengthen the idea that HTRA1 may play a negative role in bone mineral formation, thereby challenging our earlier conclusions. An important point to be clarified regarding the C3H10T1/2 osteogenesis model is that the effect of HTRA1 loss on chondrogenic differentiation during the early stages of differentiation was not investigated. Therefore, we cannot exclude the possibility that HTRA1 loss mediates its positive influence over osteogenesis through the accelerated induction of chondrocyte hypertrophy. Additional studies

investigating chondrogenic marker expression at early times points prior to day 21 would certainly help clarify this.

One possible explanation for these conflicting results is provided for by the observation that HTRA1-deficient C3H10T1/2 cells have a significantly greater tendency to undergo adipogenesis. In contrast to primary MSC cultures, including those derived from fat [315] and bone [316], the osteogenic induction of C3H10T1/2 cells is positively regulated by pro-adipogenic gene expression [314]. Indeed, the authors of this study showed that the overexpression of PPARγ2 promoted adipogenic differentiation and, surprisingly, also promoted osteogenic differentiation. Furthermore, repression of PPARγ2 consistently impaired both adipogenic and osteogenic differentiation. Therefore, it is quite possible that the stimulatory effects of HTRA1 deficiency on C3H10T1/2 osteogenesis observed in our study, were indirectly due to increases in the expression of adipogenic gene markers. The idea of loss-of-function of HTRA1 favouring adipogenesis has previously been demonstrated in hBMSCs [298,299], although, unlike HTRA1-deficient C3H10T1/2 cells, this resulted in significant reductions in their osteogenic potential [298]. Currently, it is unclear whether BMP-2 stimulated HTRA1-deficient C3H10T1/2 cells represent a heterogeneous population of adipocytes and osteoblasts, or whether they are one in the same. Their ability to temporally differentiate into all mesodermal cell types has already been reported [313,314]. Treatment of C3H10T1/2 cells with 5-Aza-CR resulted in the appearance of myocytes after 6 days. Adipocytes started to appear 4 days later, and chondrocytes appeared after an additional 9 days. Furthermore, overexpression of BMP-2 in C3H10T1/2 cells drove differentiation toward the osteogenic lineage, while BMP-4 overexpression promoted the formation of adipocytes [314]. Certainly, evidence exists to suggest that MSC-derived adipocytes have the potential to undergo trans-differentiation into osteoblasts, and even chondrocytes [317]. However, studies investigating the adipogenic and osteogenic effects of BMP-2 on C3H10T1/2 cells have so far failed to observe cells exhibiting both phenotypes simultaneously [314]. Taken together, these results further exemplify the complexities involved in trying to decipher HTRA1's role in bone formation, and provide additional support for the notion that its effects on osteogenic differentiation are predominantly cell-type specific.

Several studies have demonstrated that HTRA1 can influence TGF-β signalling, but once again, the findings are beset with inconsistencies. An *in vitro* study in

which HTRA1 was overexpressed in the myoblast C2C12 cell line demonstrated that it could degrade members of the TGF-β family such as BMP-2, BMP-4 and TGF-β1, thereby inhibiting associated downstream signalling events [233]. Additional insights into HTRA1's ability to modulate the TGF-β signalling pathway come from studies performed by Zhang *et al.* (2012), where it was demonstrated that HTRA1 could modulate angiogenesis through its interaction with TGF-β signalling members [318].

Constitutive overexpression of HTRA1 is one approach that could be taken to gain further insights into its role in C3H10T1/2 osteogenesis. Based on the findings from our *Htra1*-knockdown studies, it is assumed that long-term overexpression of HTRA1 would repress C3H10T1/2 osteogenesis and matrix mineralization. As far as we are aware, stable overexpression of HTRA1 using lentivirus transduction has been used on endothelial cells [319] and hPDLCs [320] but has never been used in MSCs. Another point that should be taken into account is the use of three dimensional (3D) cell cultures as compared to two dimensional (2D) cell culture systems, where 3D MSC cultures may provide a more physiologically relevant spatial organisation for cell-cell communication [321]. Certainly, MSC condensation is required to initiate mesenchymal differentiation. A final consideration regarding the limitations of our *in vitro* model is the fact that we investigated the role of HTRA1 in mono-cell cultures. Bone homeostasis relies on the cross-talk between osteoclasts and osteoblasts [322-324]. For this reason, it may be worth assessing the role of HTRA1 in alternative *in vitro* co-culture models. To support this idea, the role of HTRA1 has been investigated in KusaO cells as well as in primary bone-marrow macrophages, and a model of communication between bone-forming cells and bone-resorbing cells has been proposed [304].

In order to better understand the implications of HTRA1 loss on the physiology of bone formation, several research groups have generated *Htra1*-knockout mouse models. However, as with the findings from studies evaluating the effects of HTRA1 loss *in vitro*, results emanating from these *in vivo* investigations also appear to be beset by inconsistencies [262,325]. The gene trapping technique used to flox exon 2 and 3 of the *Htra1* gene is one of the models that Graham *et al.* (2013) used in their study [262]. They observed improvements in various bone structure parameters in *Htra1*-knockout mice, and concluded that this was most likely due to enhanced TGF-β signalling based on the fact that HTRA1 could

cleave TGF-β receptors *in vitro* [262]. However, in a more recent study Beaufort *et al.* (2014) [263] used another model of *Htra1*-knockout mice in which the first intron of the *Htra1* gene was trapped leading to HTRA1 transcripts lacking exons 2 to 9 by disruption of the reading frame. They showed that HTRA1 positively regulates TGF-β pathway activation *in vivo*, although its effect on bone structure was not examined. These results highlight the complex nature of trying to determine HTRA1's role *in vivo*, and suggest that inherent differences may exist between *Htra1*-knockout models.

In our study, we used an alternative *Htra1*-knockout mouse model in which *Htra1* transcription had been disrupted by the insertion of a cassette in the first exon [295]. Results from our own micro-CT analysis of intact femurs taken from 16-week-old *Htra1*-knockout mice demonstrated that their bone structure was comparable with that of age-matched wild-type mice. Moreover, despite confirming the presence of HTRA1 protein in regenerating bone in wild-type mice, and its absence from *Htra1*-knockout mouse tissue, bone repair also appeared to be unaffected by the loss of HTRA1. HTRA1's localisation at sites of, and potential involvement in, new bone formation has been attested in a recent *in vivo* study examining the effects of thyroxin exposure on calvarial growth sites in mice, where enhanced levels of HTRA1 were identified at sites of increased osteoblast activity [303]. We were therefore surprised not to have observed any significant deviations in bone regeneration in HTRA1-deficient mice. Interestingly, immunohistochemical staining of regenerating bone also identified HTRA3 at similar locations as HTRA1 in wild-type mice. Furthermore, HTRA3 was still observed in the regenerating bone tissue of *Htra1*-knockout mice. As far as we are aware, this is the first report of the presence of HTRA3 in the bone tissue of adult mice undergoing bone repair. As with HTRA1, HTRA3 was primarily detected at the borders of cartilaginous tissue within the callus, where chondrocyte apoptosis and subsequent bone remodelling are thought to occur [326]. These findings therefore signify a possible functional redundancy between these two HTRA paralogs, whereby loss of HTRA1 is compensated for by HTRA3. Further investigations into bone regeneration using mice deficient in HTRA3, or HTRA1 and HTRA3, may provide additional insights into the role or HtrA proteases in bone formation. Several knockout mice models for different HtrA members have been generated, and no obvious phenotype compared to wild-type mice has been reported [327,328]. It is widely accepted that

compensation mechanisms can occur in the case of a loss-of-function of one of the members. Indeed HTRA1 and HTRA3 share a high amino acid sequence homology [226] and it is believed that they may have comparable roles [228]. As with HTRA1, HTRA3 is localised to the placenta and has been shown to be up-regulated during placentation [248,329-331]. Additionally, and similarly to HTRA1, HTRA3 is considered to be a tumour suppressor [332,333] due to its obvious down regulation in several cancer cell lines [228,334-336]. HTRA3 can also assemble as a homotrimer, as has been reported for HTRA1 and HTRA2 [230,337]. The catalytic activity of HTRA3 has also been reported to be similar to HTRA1's cleavage specificity [226]. In addition to its N-terminal domain, HTRA3's PDZ domain has also been shown to be necessary for its proteolytic activity, as is the case for HTRA1 [229,230]. Furthermore, it has been shown that HTRA1, DDR-2 and MMP-13 are components of the same molecular pathway involved in the development of OA [259,290]. Additionally, up-regulation of HTRA1 has been linked with inflammation in OA. Increase in HTRA1 production by chondrocytes correlated with cartilage degradation *in vitro* and synovial fluid obtained from OA patients exhibited high levels of HTRA1 [296]. Furthermore, increased levels of HTRA1 correlated with increases in DDR-2 and MMP-13 expression leading to the degradation of the cell matrix [290]. Therefore, targeting HTRA1 as an upstream regulator of matrix remodelling may be one approach to treating OA [338]. In our study, we reported that *Htra1* knockdown in C3H10T1/2 cells led to a significant up-regulation of *Mmp13* whereas the immunohistochemical analysis confirmed comparable staining of MMP-13 protein *in vivo,* localized to hypertrophic chondrocytes in cartilage callus. The MMP-13 pattern *in vitro* is therefore not consistent with what's seen *in vivo*. A possible explanation for such differences is the potential compensatory role of HTRA3. Despite us being able to demonstrate HTRA3 expression in both wild-type and *Htra1*-knockout mice, we were unable to detect it in C3H10T1/2 cells. Such findings therefore imply that HTRA1 loss in C3H10T1/2 cells would not be compensated for by HTRA3, thereby rendering the cells vulnerable to alterations in osteogenic differentiation. This assumption is supported by our unpublished findings (unpublished results 4.1) where ASCs isolated from wild-type or *Htra1*-knockout mice were shown to express *Htra3*, and underwent comparable osteogenic differentiation.

Previous studies have identified HTRA1 as an inducer of premature cell senescence [339], and elevated levels of HTRA1 have been positively correlated with increased incidences of frailty in aged humans [340]. The suggested underlying mechanism linking increases in HTRA1 plasma levels with frailty is the so-called “inflammaging” [341,342]. HTRA1 is thought to bind the cytokine TGF-β1 and repress its downstream signalling, thereby promoting inflammation [233,343]. The relationship between HTRA1 and TGF-β1 has been reported in other inflammatory rheumatoid disorders [344], supporting the link between HTRA1 and cell stress such as ageing. Therefore, we also considered the possibility that changes in the bone phenotype of *Htra1*-knockout mice may become more apparent with ageing. Indeed, the bone structure in femurs of 52-week-old mice was significantly improved in *Htra1*-knockout mice as compared to their wild-type counterparts. These findings suggest that, in mice at least, HTRA1 may represent an important determining factor for bone quality in response to aging, and further studies examining bone regeneration in aged HTRA1-deficient mice may be warranted. Certainly, these new findings are more in keeping with our *in vitro* data, where mineralized matrix formation was enhanced in HTRA1-deficient C3H10T1/2 cells.

Regarding the *in vivo* evaluation of HTRA1’s effects, some perspectives are worth consideration in order to help clarify its mode of action. In bacteria, HTRA1 has been characterised as a stress protein [245], and ageing, among other things, has been intensively reported as being a stress factor [345,346]. Therefore, based on the differences observed in the bone parameters of 52-week old mice, it is possible to argue that the influence of HTRA1 on bone physiology may only be apparent in aged mice. Another, and possibly more physiologically relevant, means of assessing the influence of HTRA1 on bone parameters *in vivo* would be to produce an inducible HTRA1-deficient mouse model in which the effects of HTRA1 loss could be determined in adult mice only, thereby bypassing potential recovery mechanisms set in place from birth. Alternatively, an inducible transgenic mouse model in which HTRA1 was over-expressed could also be considered. Similarly, a HTRA1/3 double-knockout mouse model would be an ideal *in vivo* working model to assess HTRA1 and HTRA3 involvement not only in regulating bone formation, but also other tissues where they are co-localized [227,228].

However, there is still the matter of reconciling these observations with the results obtained from previous studies investigating the effects of loss or gain of HTRA1 function on MSC lineage commitment [298-301]. The choice of cell type, and the preference for immortalised cell lines over primary cells may have played some part in defining HTRA1 as a pro- or anti-osteogenic mediator. Certainly, the response of cultured cells to loss of HTRA1 varies considerably; for instance, proliferation is either decreased [347], enhanced [348], or unaffected [251], depending on the cell source used. Therefore, some caution should be taken in translating these *in vitro* findings to an *in vivo* system, in which the generation of a particular phenotype may be the culmination of a series of heterogeneous cellular responses to alterations in HTRA1 production. Taken together, our findings further identify HTRA1 as a potent regulator of the multi-lineage differentiation potential of MSCs, and provide evidence to suggest that although HTRA1 does not appear to influence bone development and regeneration beyond the *in vitro* system, it may contribute to the ageing bone phenotype in mice. Whether this is also applies to aged human bone, however, remains to be determined.

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Acknowledgements

First of all, I would like to thank PD. Dr. Peter Richards (Pete). Thanks Pete for giving me the opportunity to achieve my PhD in your lab and for fighting until the end to make the best out of our work despite an unusual and unforeseen situation.

I am very thankful to Prof. François Verrey who accepted to be the chairman of my PhD committee. Your extensive experience, profound knowledge, availability and sympathy made you a trustworthy person and someone I could always rely on. I was lucky and honoured to be evaluated by such a remarkable individual.

Many thanks to PD. Dr. Paolo Cinelli, Prof. Franz Weber and Dr. Jerôme Lafont for being a part of my PhD committee. Your expertise, valuable insights, and involvement guided my work throughout these 3 years. I wish every PhD student to have the same stimulating and supportive committee.

I am grateful to have crossed paths with all my lab mates while completing my PhD. With the years they did not only were my colleagues but also became my friends. Nicki was a daily support; his pedagogy, patience and great sense of humor were of valuable help. Nicki, no matter what was going on, I cannot remember a day without laughing when you were around, I don't know WHY! Ali, I definitely could not have made it without you. Your considerable help on this project was really precious to me and I could never thank you enough. Greg and Stephan, my 2 lab big brothers, taught me grumpiness and my first German words by making me listen to traditional German songs. I admit, you guys initiated my German integration! Coqui, there is so much to say about you. Everything started in the lab or during shoe shopping, I don't remember. From then on we became friends and roommates. You finally were crazy enough to offer me to be your Bridesmaid on your wonderful Puerto Rican wedding. The first highlight of my year 2017! I also would like to thank Silke and Marina, for sorting out so many things for me. Your kindness and help made paperwork so much nicer!

Thank you to Pauline and Kasia, the lab girls! I am really happy that I met both of you. Working days are so much better with an overdose of Maroon 5 songs! I hope we keep in touch after the lab times!

During my education, I had the chance to meet great people who contributed to my scientific education. My first lab experiences, the CRCL (Harlem Shake!) and then the CRCM, who convinced me to continue in science. McGill and my "québecois" trip: it was the coldest period of my life, but felt so much warmer surrounded by the amazing people I met. Thank you!

I will never be able to thank my parents enough for their boundless love and support. Dad, you wrote in your own thesis that you wished for your work to prepare my future, I hope your expectations are fulfilled. I admire my Mom for her determination and strength of mind; she would have stopped the world from spinning for me to succeed. Mom, no matter how old I get, you will always remain my example of bravery and loyalty. Baptiste, my brother I love so much. I wish you to find the way (not in Academia!) that makes you happy. You can

count on me to give my opinion and advice; I will always be by your side as your big sister. I am so happy that I belong to this crazy family. They have given me a solid foundation in life and I know that when I am with them, I experience real happiness.

To Philip and his unconditional love, support and listening. I could always count on you in troubled or wonderful times. You gave me the strength and motivation to accomplish things beyond what I could ever imagine. Thank you for being such a wonderful person.

Thank you, Romain, for those thousands of "Pskypanalysis" about PhD, life and other foolish topics. I hope you know how much your friendship and support are precious to me and were valuable help during these 3 years. Bil, my adventure friend, always calm, kind and thoughtful. We have so many memories together that make me smile every time I think about them.

Emilie my heart-sister and Laurent with whom I always have great times. You entrusted me to be the Godmother of their beautiful son Anton, and the witness of your upcoming wedding. These were the second and third highlights of 2017. Next to these responsibilities, doing a PhD did not seem to be that impossible after all.

I would like to thank all my friends from Saint-Cham Beach Camille(s), Amélie, Tiphaine, Steph, Denis, Sandy, Pichet, Julie and the others that belong to our big "St-Cham " group. All my friends I met at the University in Saint-Etienne and Lyon with whom I shared so many crazy and funny moments.

To Johanna, Elodie, Silvia, Silke and Elizabeth for being my closest friends in Zürich. Thank you for the fun times, for your kindness and for all the wonderful and precious moments we shared. I hope we will continue this way!

Robert (Bob), Chafik, Nicki and Stephan for their help and generosity, especially with my manuscript.

Thanks to all the people I could not cite here but who contributed in many ways to make this experience intense and fun, graduating and closing the chapter of my academic education is the fourth highlight of 2017!

Curriculum Vitae

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Working experience

❖ **CABMM** **2014 - Present**

PhD Student

Centre of Applied Biotechnology and Molecular Medicine - University of Zürich – Switzerland (Peter J. Richards, PD)

During my PhD I acquired strong knowledge in different areas such as generating outcomes, analytical thinking, organising and troubleshooting. I learnt to sharpen my critical thinking to efficiently develop my project. I also experienced teaching by assisting practical classes and developed my presentation skills to successfully communicate an idea.

❖ **CABMM** **March to June 2014**

Intern

Centre of Applied Biotechnology and Molecular Medicine - University of Zürich – Switzerland (Peter J. Richards, PD)

I contributed to the establishment of cell culture models (3D and 2D) to investigate the involvement of the serine protease HTRA1 in bone formation and its ability to influence BMSCs differentiation through chondrogenesis by using RT-qPCR, transfection and lentivirus transduction. There, I successfully initiated a new project and started to set up the basis required for the beginning of my PhD.

❖ **ENTR'AID** **2012 - 2013**

Vice-president

University-associated charity association. Fund-raising in favour of Ehlers-Danlos patients, University of Lyon – France

Association created with friends meant to organise cultural and sports events on the campus. I got familiar with finding sponsors, dealing with administrations and official authorisations, communicate and promoting an event, managing a budget, respect deadlines and negotiate an offer. The funds collected were donated to the French Association of Ehlers-Danlos patients to purchase medical devices.

❖ **MUHC** **October to January 2013/14**

Intern

McGill University Health Centre, Montreal – Canada (Jean-Jacques Lebrun, Pr).

I successfully established a stable melanoma cell lines overexpressing a neural transcription factor, Neurogenin 2 to investigate the role of TGFβ in skin cancer context. I achieved my objectives by using PCR, cloning and lentivirus transduction technics.

❖ **CRCM** **July to August 2013**

Intern

Cancer Research Centre of Marseille, University of Marseille – France (Patrice Dubreuil, PhD).

I volunteered for an internship based on the functional identification of activating mutations in tyrosine kinase receptors Platelet-Derived Growth Factor Receptor by using Western-blotting analysis.

❖ **CRCL** **January to March 2013**

Intern

Cancer Research Centre of Lyon, University of Lyon – France (Colette Roche, CR).

My work involved immunohistochemistry, Western-blotting and q-PCR analysis of the human Semaphorin 3F overexpressed in different cell lines. These *in vitro* experiments were completed with *in vivo* investigations by staining mice liver sections exhibiting neuron-endocrine tumours or not.

Education

❖ **TRREE Certifications** **2017**

Training and Resources in Research Ethics Evaluation.

Good Clinical Practice (**GCP**), HIV Vaccine Trials, Adolescent Involvement in HIV Prevention Trials, Public Health Research Ethics, Informed Consent, Research Ethics Evaluation, Introduction to Research Ethics, Swiss National Supplement.

❖ **CABMM** **2014 - Present**

PhD Student

Centre of Applied Biotechnology and Molecular Medicine - University of Zürich – Switzerland.

My PhD project consisted in investigating the role of the serine protease HTRA1 in bone formation and regeneration. To achieve my work I worked *in vitro* with different cell lines and technics of culture, transfection, lentivirus transduction, RT-qPCR, straining for cells and sections, protein purification on Åkta system. I also used an *in vivo* femur osteotomy model to investigate bone healing in *HtrA1*-knock-out and wild-type mice.

❖ **HEALTH CARE DIAGNOSTICS IN 2025** **August 2016**

Summer School, Ecole Polytechnique Fédérale de Lausanne.

Selected for a one-week summer school, I got the opportunity to discuss how science is going to be led within the next years, its impact on populations and how scientists will have to adapt to technological, economical and social perspectives.

❖ **MASTER** **2012 - 2014**

Genetics, cell biology and pathology - Claude Bernard University, Lyon – France.

During my Master degree, I deepened my skills in cell biology, molecular biology, genetics, analysis of articles, genomics, immunobiology, introduction to project management. My academic education was strengthened by practical courses and internships abroad.

❖ **BACHELOR** **2009 - 2012**

Genetics and cell biology - Claude Bernard University, Lyon – France.

My bachelor degree specialized me in cell biology, physiology, microbiology, genomics, biochemistry, chemistry and biophysics.

❖ **ENSASE** **2008 - 2009**

National School of Architecture, Saint-Etienne – France.

A year of architecture school taught me how to think and design a project, materials strength, project execution.

Personal interests

❖ **HORSE RIDING** **2001 - 2005**

During my studies I practiced top-level horse riding by joining a specific sport-school program.

❖ **DRAMA CLASSES** **2005 - 2013**

I attended private drama classes, played in an amateur company that aimed to collect funding for charity organisations. In 2007, I had the chance to be a part of a one-week program at the Cours Florent (one of the most prestigious drama school in France).

❖ **ASSOCIATION DE CHAVANNE**

Member of a charity and social organization member in my hometown. We organize hiking and biking tours, Christmas market, and activities for children and adults. Those activities aim to entertain and gather people from the same city.

❖ **TRAVELLING**

Discovering is an important part of my personal interests. Learning from different cultures is my way to stay open-minded.

List of publications

❖ **Gladys Filliat**, Ali Mirsaidi, André N. Tiaden, Gisela A. Kuhn, Franz E. Weber, Chio Oka, Peter J. Richards, *Role of HTRA1 in bone formation and regeneration: In vitro and in vivo evaluation*, Plos One, 2017

❖ Stephan Glanz, Ali Mirsaidi, Cristina López-Fagundo, **Gladys Filliat**, André N. Tiaden, Peter J. Richards, *Loss-of-Function of HtrA1 Abrogates All-Trans Retinoic Acid-Induced Osteogenic Differentiation of Mouse Adipose-Derived Stromal Cells Through Deficiencies in p70S6K Activation*, Stem Cells Dev, 2016.